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Graded Reoxygenation in the Resuscitation of Asphyxiated Newborns

by

Erika Haase

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A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Master of Science

in

Experimental Surgery

Department of Surgery

Edmonton, Alberta
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ABSTRACT

Perinatal asphyxia may lead to shock and hypoxic-ischemic injury. Resuscitating asphyxiated newborns will create a hypoxia-reoxygenation process that may cause further clinical complications. We hypothesized that lower oxygen concentrations may be safe and effective in newborn resuscitation. The objective was to examine the pathophysiologic effects of hypoxia-reoxygenation with different oxygen concentrations. Newborn piglets subjected to severe hypoxemia for two hours were resuscitated with 21%, 50% or 100% oxygen while systemic and regional hemodynamic parameters were measured. Piglets resuscitated with 21% oxygen showed improved recovery of cardiac output and superior mesenteric artery flow, and elevated carotid artery flow during early reoxygenation. Resuscitation with 100% oxygen resulted in depletion of myocardial glutathione levels, which correlated with cardiac function, and intestinal glutathione oxidation and tissue injury. We conclude that 21% oxygen is safe and effective for resuscitation of asphyxiated newborns and may be superior to 100% oxygen.

To my parents,
for helping me to reach all my goals and dreams.

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List of Abbreviations

AMP	Adenosine 5'-monophosphate
ANOVA	Analysis of variance
ATP	Adenosine 5'-triphosphate
BPD	Bronchopulmonary dysplasia
C_aO_2	Arterial oxygen content
CI	Cardiac index
CLD	Chronic lung disease
CVP	Central venous pressure
DO_2	Oxygen delivery
F_iO_2	Fraction of inspired oxygen
GSH	Glutathione
GSH-Px	Glutathione peroxidase
GSNO	S-nitrosoglutathione
GSSG	Glutathione disulfide (oxidized glutathione)
GSSG:GSH	Glutathione redox ratio
H_2O_2	Hydrogen peroxide
HOCl	Hypochlorous acid
HR	Heart rate
HX	Hypoxanthine
LOOH	Lipid hydroperoxides
MAP	Mean arterial pressure
MPO	Myeloperoxidase
NEC	Necrotizing enterocolitis
NO	Nitric oxide
$O_2^{\bullet}$	Superoxide radical
$OH^{\bullet}$	Hydroxyl radical
OFR	Oxygen free radical
$ONOO^-$	Peroxynitrite anion

P_aCO_2	Arterial partial pressure of carbon dioxide
P_aO_2	Arterial partial pressure of oxygen
PAP	Mean pulmonary arterial pressure
PVRI	Pulmonary vascular resistance index
RM ANOVA	Repeated measures analysis of variance
SMA	Superior mesenteric artery
SOD	Superoxide dismutase
SVI	Stroke volume index
SVRI	Systemic vascular resistance index
XD	Xanthine dehydrogenase
XO	Xanthine oxidase

CHAPTER 1

Perinatal Asphyxia

The transition from fetal to neonatal life is accompanied by considerable physiologic changes in the newborn. Perinatal asphyxia can occur during this critical period, and may result in a failure to adapt to these physiologic stresses. Asphyxia is defined as hypoxia in the presence of acidosis.[1] In the perinatal period, asphyxia can be a result of complications such as inadequate maternal oxygenation or perfusion pressure, or inadequate blood delivery to the fetus due to umbilical cord compression, excessive uterine contractions, placental separation, or placental insufficiency, (see table 1-1). After birth, hypoxia and asphyxia may result from severe anemia, shock, overwhelming sepsis, respiratory depression, or congenital anomalies interfering with respiratory or cardiovascular function.[1]

Perinatal asphyxia is a common clinical condition, occurring in 3% to 5% of deliveries,[2] with 4 to 7 million newborn infants worldwide requiring some form of resuscitation immediately after birth.[3] The most severe cases of perinatal asphyxia may be fatal, with 1 million deaths occurring worldwide each year.[4] In Canada, the neonatal death rate is 3.9 per 1000 live births, many of which are related to perinatal asphyxia.[5]

Significant morbidity is also associated with perinatal asphyxia due to hypoxic-ischemic injury to major organ systems. The degree of organ injury is determined by the severity and duration of asphyxia, as well as selective circulatory adjustments which occur.

TABLE 1-1. RISK FACTORS FOR PERINATAL ASPHYXIA

Antepartum risk factors	Intrapartum risk factors
Multiple gestation	Pre- or post-term gestation
Maternal bleeding	Fetal distress
Premature rupture of membranes	Meconium-stained amniotic fluid
Eclampsia	Prolapsed cord
Decreased fetal activity	Abruptio placenta
Maternal hypertension	Placenta previa
Maternal diabetes	Uterine tetany
Maternal sexually transmitted disease	Narcotic administration to mother
Abnormal amniotic fluid volume	Emergency caesarean section
Substance abuse	Abnormal presentation
Poor prenatal care	Forceps or vacuum delivery
Congenital malformation	Chorioamnionitis
Chronic placental insufficiency	Prolonged labour

Circulatory Redistribution in Hypoxemia and Asphyxia

During hypoxemia and acidosis, circulatory adjustments occur which differentially effect blood flow and oxygenation to different organs, preferentially shunting blood to the more vital organs for survival.[6-8] This occurs by three main mechanisms: local vascular effects, adrenergic stimulation, and chemoreceptor stimulation.

Local vasodilation occurs when vascular beds are perfused with hypoxic blood. Vasodilation is seen predominantly in the cerebral and coronary vasculature, in contrast to skeletal muscle which vasodilates only minimally, and the renal vasculature which does not vasodilate even at very

low oxygen tension.[7]

In response to perinatal stress such as hypoxia or asphyxia, sympathetic nervous system activation and catecholamine release result in alpha-adrenergic receptor-mediated vasoconstriction and beta-adrenergic receptor-mediated vasodilation in the peripheral circulation. In some vascular beds such as the splanchnic circulation, kidneys and skeletal muscle, the alpha-adrenergic response predominates, leading to overall vasoconstriction in these vascular beds during asphyxia.[7,9,10]

Chemoreceptor stimulation in response to a decrease in P_aO_2 and pH causes a local vasoconstriction in an attempt to increase central arterial blood pressure. The chemoreceptor response is seen in skeletal muscle and bowel, but there is little response in the cerebral, coronary or adrenal circulation.[7]

The overall effect of local vascular effects, adrenergic stimulation and chemoreceptor stimulation results in vasodilation of cerebral and coronary vascular beds and vasoconstriction of splanchnic, renal, and skeletal muscle beds, in an attempt to preserve blood flow to the most metabolically active organs.[6,7] This selective redistribution of blood flow may result in hypoxic-ischemic injury to less vital organs. Furthermore, this protective reflex has a limited capacity, and if the asphyxial insult is severe and prolonged enough, the redistribution of blood flow will be inadequate to meet the metabolic needs of those vital organs. Thus, in perinatal asphyxia, all organ systems are at risk for sustaining hypoxic injury.[8]

Scope of Multisystem Involvement in Perinatal Asphyxia

Dysfunction of one or more organ systems occurs in 82% of infants following perinatal asphyxia.[11] The central nervous system is the most frequently involved (72%), followed by kidney (42%), heart (29%), intestine (29%), and lung (26%). Table 1-2 describes the clinical and pathological effects of asphyxia on different organ systems.

Central Nervous System. Perinatal asphyxia is the single most important cause of brain injury in newborns.[12] Hypoxic-ischemic encephalopathy may manifest in the newborn as lethargy, abnormal tone and reflexes, seizures, and coma, often within the first 24 hours of life. After 2-3 days, infants may develop cerebral edema and increased intracranial pressure as a result of delayed neuronal damage.[12,13] Depending on the severity, 25 to 30% of infants with hypoxic-ischemic encephalopathy may go on to develop permanent neurologic sequelae.[1]

Cardiovascular System. Perinatal asphyxia usually causes immediate cardiovascular changes including hypotension and bradycardia, necessitating urgent resuscitation at birth.[1] Later, myocardial ischemia may manifest clinically with impaired myocardial contractility and ongoing hypotension. Primhak *et al.*[14] studied cardiac effects in 20 term newborns with asphyxia and demonstrated a correlation between clinical myocardial dysfunction, EKG changes, and elevated levels of the myocardial fraction of creatine kinase (CK-MB). Five of twenty infants in this study died and all were

TABLE 1-2. MULTISYSTEM EFFECTS OF PERINATAL ASPHYXIA

Organ system	Effect
Central nervous system	Hypoxic-ischemic encephalopathy Intracranial hemorrhage Stroke Seizures Cerebral edema Hypotonia, hypertonia
Cardiovascular	Myocardial ischemia Cardiogenic shock, heart failure, cardiac stunning Hypotension Tricuspid regurgitation Dysrhythmias Bradycardia Patent ductus arteriosus
Pulmonary	Pulmonary hypertension Hyaline membrane disease Pulmonary edema Pulmonary hemorrhage
Gastrointestinal	Bowel perforation, ulceration, hemorrhage, necrosis Necrotizing enterocolitis
Liver	Cholestasis Hepatitis
Renal	Acute tubular or cortical necrosis Acute renal failure
Adrenal	Adrenal hemorrhage
Metabolic	Hypoglycemia Hypocalcemia Lactic acidosis
Hematology	Disseminated intravascular coagulation Thrombocytopenia

found to have myocardial necrosis at autopsy. The cardiovascular effects of acute asphyxia have been studied in animal models. Randall[15] showed that term fetal pigs asphyxiated *in utero* by umbilical cord occlusion developed an initial profound drop in heart rate in the first 3 minutes followed by transient recovery then gradual decline until asystole by 22 minutes. Westgate *et al.*[16] and Gunn *et al.*[17] demonstrated in fetal lambs that repeated, intermittent umbilical cord occlusion resulted in bradycardia, hypotension, and ischemic ST-segment changes on electrocardiogram, as well as subendocardial injury and papillary muscle necrosis. The degree of histologic injury correlated with hemodynamic evidence of myocardial stunning, indicated by a delay in recovery of mean arterial pressure and heart rate for 12 hours after blood flow was restored.[17]

Pulmonary System. Respiratory distress in newborns with perinatal asphyxia is multifactorial. The passage of meconium is a sign of fetal distress, and may result in meconium aspiration syndrome which is an important cause of respiratory distress in asphyxiated newborns, in the presence of meconium-stained amniotic fluid.[18] Also, surfactant deficiency or dysfunction can occur in the setting of perinatal asphyxia, resulting in hyaline membrane disease which occurs in 60-80% of premature infants and 5% of term newborns.[18] Infants with hyaline membrane disease develop interstitial edema, hyaline membrane formation, and reduced lung compliance, which lead to hypoxemia, increased pulmonary vascular resistance, and persistent fetal circulation. Premature neonates with severe hyaline membrane disease require prolonged intubation, high inspired oxygen concentrations, and often

progress to chronic lung disease (bronchopulmonary dysplasia).[18]

Persistent pulmonary hypertension of the newborn may develop as an associated feature of hyaline membrane disease, meconium aspiration syndrome, pneumonia, or congenital diaphragmatic hernia. The common etiology of persistent pulmonary hypertension of the newborn is hypoxemia, which leads to an increase in pulmonary vascular resistance and pulmonary hypertension. This in turn leads to increased right heart pressures and the development of right→left shunting through the foramen ovale and ductus arteriosus which are opened by the pressure gradient. This is referred to as persistent fetal circulation, which causes further hypoxemia which is often resistant to inhaled oxygen.[19,20]

Gastrointestinal System. Necrotizing enterocolitis (NEC) is the most common gastrointestinal emergency in neonates with an overall incidence of 1.1 per 1000 live births.[21,22] The incidence of NEC has been increasing as a result of improvements in neonatal intensive care.[22,23] The mortality associated with NEC has been reported from 10 to 66%, and those that survive are at risk for long term complications such as short bowel syndrome, intestinal strictures and obstruction.[21-24] Ninety percent of cases occur in premature infants, at a rate of 11.4/1000 live births in infants with birth weights less than 1000 grams, compared to 0.1/1000 in infants greater than 2500 grams or greater than 38 weeks gestational age.[21] NEC is characterized by abdominal distension and tenderness, occult or frank blood in stools, intestinal gangrene, pneumatosis intestinalis, bowel perforations, sepsis and shock. Bell *et al.*[25] proposed a staging system for the severity of NEC based on clinical

and radiologic criteria (see table 1-3). Stage I represents nonspecific clinical and radiologic signs which should lead the clinician to suspect NEC, although similar findings may be seen with sepsis, gastroenteritis, simple ileus or feeding intolerance, or hypoglycemia.[22,26] Stage II and III represents definitive NEC with radiologic or pathologic evidence.

TABLE 1-3. BELL STAGING SYSTEM FOR NECROTIZING ENTEROCOLITIS (NEC)

Stage	Classification	Systemic signs	Intestinal signs	Radiologic signs
I	**Suspected NEC**	Temperature instability, apnea, bradycardia, lethargy	Increased gastric residuals, mild abdominal distension, emesis, occult blood-positive stools or bright red blood per rectum	Normal or intestinal dilation, mild ileus
II	**Proven NEC** *mild to moderately ill*	Same as above plus mild metabolic acidosis and thrombocytopenia	Same as above plus absent bowel sounds, abdominal tenderness	Intestinal dilation, ileus, pneumatosis intestinalis, ascities
III	**Advanced NEC** *severely ill*	Hypotension, bradycardia, severe apnea, combined metabolic and respiratory acidosis, disseminated intravascular coagulation and neutropenia	Generalized peritonitis, marked tenderness and abdominal distension	Same as above plus pneumo-peritoneum

The etiology of NEC has not yet been fully established, but is likely multifactorial. Several studies have reported NEC is associated with low birth weight, perinatal asphyxia and respiratory distress,[27-29] and pathologic analysis of intestinal specimens of NEC demonstrate ischemic necrosis identical to other ischemic intestinal insults.[30] Furthermore, several newborn animal studies have demonstrated that severe hypoxia or asphyxia can results in intestinal lesions similar to those seen in NEC.[31-34]

Renal System. Perinatal asphyxia causes acute tubular necrosis and renal failure in 9 to 30% of asphyxiated newborns.[1] Severely asphyxiated neonates with renal failure have been shown to develop an elevation in plasma creatinine during the first 5 days of life, oliguria unresponsive to fluid challenge, and decreased renal blood flow velocity.[35,36] Renal function and plasma creatinine have been shown to normalize within the first 2 weeks of life in most asphyxiated infants.[36]

Conclusion

Perinatal asphyxia is potentially fatal, and in survivors, severe asphyxia often causes devastating injury to multiple organ systems. It is important to optimally manage asphyxiated infants, beginning with neonatal resuscitation, in order to prevent organ injury and potential long term sequelae.

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CHAPTER 2

Neonatal Resuscitation with 21% versus 100% Oxygen

Contemporary guidelines for neonatal resuscitation recommend that 100% oxygen be administered to the asphyxiated newborn showing any signs of respiratory distress, cyanosis or apnea,[1,2] as stated in the most recent American Heart Association and American Academy of Pediatrics guidelines:

> *"If assisted ventilation is required, deliver 100% oxygen by positive-pressure ventilation. If supplemental oxygen is unavailable, initiate resuscitation of the newly born infant with positive-pressure ventilation and room air. The goal of supplemental oxygen use should be normoxia."*[3]

However these recommendations are based on historical practice rather than scientific evidence that supports the use of 100% oxygen in newborn resuscitation.[4] In recent decades, an increasing awareness of oxygen toxicity and oxygen radical diseases of the newborn has led researchers and clinicians to question the use of 100% oxygen in neonatal resuscitation.

In 1993 a pilot study was published which prospectively compared the use of 21% versus 100% oxygen in the resuscitation of 84 asphyxiated newborns (heart rate < 80 and/or apnea at birth requiring resuscitation).[5] They found no difference in mortality nor major neurologic impairment between the two groups. Heart rate recovered equally well in both groups, and 5-minute Apgar scores were significantly higher in the 21%-resuscitated group. The same group then continued with a prospective, randomized, multicentre study, comparing 288 asphyxiated newborns resuscitated with 21% oxygen versus 321 resuscitated with 100% oxygen.[6] They demonstrated no difference in neonatal mortality nor the incidence of moderate or severe

hypoxic-ischemic encephalopathy. Furthermore infants resuscitated with 21% oxygen had higher 1-minute Apgar scores; earlier first breath and first cry, and no difference in acid-base status or heart rate compared to the 100% group. They concluded that room air is at least as effective as 100% oxygen in the resuscitation of asphyxiated newborns.

Another group reported similar findings in a prospective, randomized, blinded study comparing the use of 21% versus 100% oxygen in the resuscitation of 40 asphyxiated newborns (hypotonia, apnea, unresponsive, bradycardia, hypoxemia, hypercarbia and acidosis).[7] They found that the time needed for the onset of a sustained respiratory pattern was significantly shorter, and the onset of the infant's first cry was significantly earlier in the 21% resuscitation group. They also examined biochemical evidence of oxidative stress, as measured by the level of oxidized glutathione (GSSG), and the antioxidant enzymes superoxide dismutase, catalase, and glutathione peroxidase. They found that infants in the 100%-resuscitation group had evidence of oxidative stress as indicated by increased blood GSSG and red blood cell antioxidant enzymes at 28 days post-resuscitation, compared to a group of non-asphyxiated controls. Levels of GSSG and antioxidant enzymes in the 21%-resuscitation group at 28 days were similar to control values. They concluded that exposure to high oxygen concentrations, even briefly during neonatal resuscitation, could lead to prolonged biochemical evidence of oxidative stress in the newborn.

In order to develop a better understanding of the acute physiologic and biochemical changes to the organism during hypoxemia and

resuscitation, animal models have been developed. Saugstad and colleagues have studied the effects of hypoxia (inspired oxygen concentration of 8% until hypotension, bradycardia, or severe metabolic acidosis developed) and reoxygenation with 21% versus 100% oxygen in newborn piglets. In 1992 Rootwelt *et al.*[8] reported no difference in mean arterial pressure, heart rate and base deficit in piglets resuscitated with 21% oxygen for 20 minutes compared to 100% oxygen. In 1993, Rootwelt *et al.*[9] reported that regional cerebral blood flow to the forebrain, midbrain, brainstem, cerebellum, and cervical spinal cord increased similarly in piglets resuscitated with 21% or 100% oxygen during the first 20 minutes of reoxygenation. Rootwelt *et al.*[10] later described regional blood flow to multiple organs (heart, kidney, intestine, liver, pancreas, spleen, muscle) in response to asphyxia and reoxygenation with 21% or 100% oxygen, as measured by the radioactive microsphere technique. They found no significant differences between 21%- and 100%-resuscitated piglets in regional blood flow to any organ at any time. However in their study, cardiac output did not significantly decrease during hypoxia, thus the severity or duration of asphyxia may have been insufficient to produce significant changes in perfusion during reoxygenation. Pulmonary and systemic hemodynamics in the same experimental model were reported by Medbo *et al.*[11] They observed hypoxia-induced pulmonary hypertension in asphyxiated piglets, but no difference in pulmonary artery pressure or pulmonary vascular resistance was seen during reoxygenation with 21% versus 100% oxygen. Cardiac index was significantly greater in the 21% group at 5 minutes of

reoxygenation.

Tollofsrud *et al.*[12] studied the effects of hypoxia followed by severe meconium aspiration prior to reoxygenation with 21% or 100% oxygen. They found no significant differences between the 21%- and 100%-resuscitated piglets during 120 minutes of reoxygenation in mean arterial pressure, pulmonary arterial pressure, cardiac index or base excess, and concluded that 21% oxygen is as efficient as 100% in the resuscitation of hypoxic piglets with meconium aspiration.

Temesvari *et al.*[13] developed a model of asphyxia induced by bilateral pneumothoraces in 3- to 6-hour old piglets. After approximately one hour piglets developed severe respiratory distress, the pneumothoraces were relieved, and piglets were resuscitated with either 21% or 100% oxygen for 10 minutes, and then were able to spontaneously breath room air during recovery for 3 hours. They demonstrated that asphyxiated newborn piglets resuscitated with 100% oxygen had significantly worse early clinical neurologic outcome compared to both 21% piglets and controls. Cerebral histopathology revealed marked damage of similar severity in both asphyxiated groups.

Conclusions

Studies in both humans and animals support the suggestion that 21% oxygen is as effective as 100% oxygen in the resuscitation of asphyxiated newborns. However, the complex pathophysiologic changes occurring

during hypoxia and reoxygenation are not yet fully understood. Ongoing clinical and basic science research will help to elucidate the pathophysiology of hypoxia-reoxygenation injury, and to strengthen the evidence for the use of appropriate oxygen concentrations during newborn resuscitation.

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CHAPTER 3

Oxygen Free Radicals and Antioxidant Defences in Newborns

Perinatal asphyxia can lead to tissue hypoxia in which oxygen delivery is inadequate for normal cellular metabolism.[1] Recovery from the hypoxic insult necessitates a prompt restoration of oxygenated blood flow. Paradoxically, tissue injury occurs predominantly not during hypoxia, but instead during the reoxygenation phase, when molecular oxygen is reintroduced to the tissues.[2] This "oxygen paradox" has been recognized since the 1950's,[3] and in recent decades has been attributed to the generation of oxygen free radicals (OFRs).[1,2,4] These cytotoxic and highly reactive molecules have been implicated in the pathogenesis of a variety of diseases in newborns, including chronic lung disease, retinopathy of prematurity, periventricular leukomalacia, necrotizing enterocolitis, and patent ductus arteriosus.[5,6] A thorough understanding of the pathophysiology of hypoxia-reoxygenation is necessary to fully understand the clinical consequences of perinatal asphyxia, and to prevent some of these devastating sequelae.

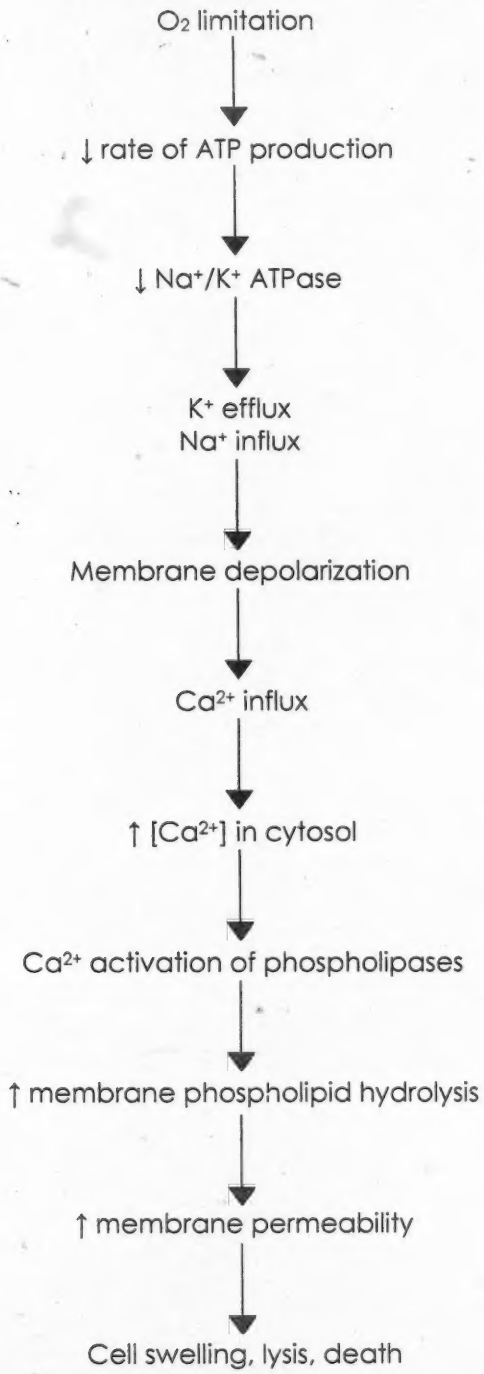
HYPOXIA

When tissue oxygen supply is limited, a series of biochemical events occurs which lead to cellular and membrane dysfunction, intracellular edema and cell death.[2] Oxygen is the basic cellular fuel essential for most cell functions. Under normal conditions aerobic metabolism occurs via oxidative phosphorylation, which allows for efficient generation of high energy phosphates, primarily adenosine 5'-triphosphate (ATP), which is

necessary for normal cellular function.[1] Under conditions of tissue and cellular hypoxia, the cell switches to anaerobic glycolysis which is a less efficient mechanism of generating energy substrate, producing only 6% as much ATP per molecule of glucose compared to aerobic metabolism.[1,2,7] Furthermore, during anaerobic metabolism, glucose is metabolized to pyruvate and lactate, thereby contributing to the metabolic acidosis seen in perinatal asphyxia.

During anaerobic glycolysis, ATP is rapidly depleted as demand for ATP outweighs its supply, (see figure 3-1). Depletion of cellular energy stores leads to an inability to maintain transmembrane ionic gradients due to decreased function of plasma membrane ion pumps including the sodium-potassium-ATPase (Na^+/K^+ATPase).[8] This results in efflux of potassium ion and influx of sodium ion which causes membrane depolarization, causing voltage-dependent calcium channels to open and an increased influx of calcium ion (Ca^{2+}).[9] Higher intracellular concentrations of Ca^{2+} activate membrane phospholipases which catalyze hydrolysis of cell-membrane and organelle phospholipids.[7,9] Activation of mitochondrial phospholipases disrupts oxidative phosphorylation, further reducing ATP production.[2] Unable to maintain homeostasis, damage to the cell membrane and increased sodium influx lead to fluid movement into the cell, cell swelling, lysis, and death.[2,7]

FIGURE 3-1
SEQUENCE OF METABOLIC EVENTS DURING HYPOXIA.



REOXYGENATION

In order for the neonate to survive a hypoxic insult, correction of hypoxia is necessary before massive cellular and tissue destruction occurs. This can only be accomplished by the reintroduction of oxygen and adequate blood flow, to both restore the energy supply and remove toxic metabolites.[2] Although tissue damage can occur during hypoxia, more severe injury can occur during this reoxygenation phase. It is now well-established that the primary mediators of reoxygenation injury are OFRs.[1,2,4,10]

Oxygen Free Radicals

Oxygen free radicals, are highly unstable, highly reactive molecules containing one or more unpaired electrons, produced by partial reduction of molecular oxygen (O_2). Under normal conditions over 95% of O_2 is reduced tetravalently to water (four electrons are donated to O_2 molecule in a single process) by the cytochrome system in mitochondria as a normal part of oxidative metabolism, (see figure 3-2).[10-12] However even under physiologic conditions, a small proportion of O_2 is reduced by a sequential univalent pathway via mitochondrial, endoplasmic reticular and nuclear membrane electron transport processes, and soluble proteins and oxidases, leading to the formation of OFR intermediates.[10,11,13-15] Univalent reduction of O_2 forms the superoxide radical ($O_2^{\bullet}$), catalyzed by the enzyme xanthine oxidase (XO). Superoxide on its own has relatively low cytotoxicity, however the main consequence of initiating the univalent reduction of O_2 is the

FIGURE 3-2

OXYGEN REDUCTION REACTIONS AND OXYGEN FREE RADICAL GENERATION

Tetravalent reduction of oxygen to water

$$O_2 + 4H^+ + 4e^- \longrightarrow 2\,H_2O$$

Univalent reduction of oxygen to form superoxide radical ($O_2^{\bullet}$), catalyzed by xanthine oxidase (XO)

$$O_2 + e^- \xrightarrow{XO} O_2^{\bullet}$$

Divalent reduction of oxygen to form hydrogen peroxide (H_2O_2)

$$O_2 + 2e^- + 2H^+ \longrightarrow H_2O_2$$

Dismutation of superoxide to hydrogen peroxide, catalyzed by superoxide dismutase (SOD)

$$O_2^{\bullet} + e^- + 2H^+ \xrightarrow{SOD} H_2O_2$$

Iron-catalyzed Haber-Weiss (Fenton) reaction

$$O_2^{\bullet} + H_2O_2 \xrightarrow{Fe^{2+/3+}} OH^{\bullet} + OH^- + O_2$$

initiation of a series of reactions which yield more potent radical species. The superoxide molecule undergoes dismutation to hydrogen peroxide (H_2O_2), catalyzed by the endogenous enzyme superoxide dismutase (SOD). Alternately, O_2 can undergo divalent reduction directly to H_2O_2. Molecular oxygen cannot be directly reduced by three electrons, but $O_2^{\bullet}$ and H_2O_2 react in the iron-catalyzed Haber-Weiss (Fenton) reaction to yield the extremely reactive and cytotoxic hydroxyl radical ($OH^{\bullet}$).[10,14] Superoxide can react with the Fe^{3+} form of iron stored in ferritin to liberate Fe^{2+}, which then reacts with H_2O_2 to form $OH^{\bullet}$.[16]

Sources of OFRs during hypoxia-reoxygenation

In contrast to the minimal physiologic production of OFRs during normal mitochondrial oxidative metabolism, under pathologic conditions such as hypoxia-reoxygenation, there is a burst of OFR formation.[1,2,4] The most essential component for OFR generation is molecular oxygen. Korthuis *et al.*[17] demonstrated that reperfusion of ischemic skeletal muscle with hypoxic blood (P_aO_2 3 to 5 mmHg) significantly attenuated reperfusion injury, and Parks and Granger[18] obtained a similar result upon reperfusion of intestine with normal saline. These experiments confirmed that oxygen is indeed an essential substrate for OFR-mediated injury. Four main sources of free radical generation in hypoxia-reoxygenation have been recognized: (1) the xanthine oxidase-hypoxanthine system, (2) nitric oxide-derived free radicals, (3) neutrophil peroxidases, and (4) the mitochondrial electron transport chain.

Xanthine Oxidase system

Xanthine oxidase (XO) is the most well-documented biological source of OFRs, and has been detected in most animal species.[11,19] Xanthine oxidase is responsible for the generation of $O_2^{\bullet}$ and H_2O_2, which then react in the presence of iron to form the highly toxic $OH^{\bullet}$ radical.[19] Hypoxia sets the stage for XO-mediated reoxygenation injury by the production of both the substrate (hypoxanthine, HX) and the enzyme (XO) necessary for OFR generation.[4] As shown in figure 3-3, during hypoxia ATP is rapidly utilized as a cellular energy source, and is broken down to adenosine 5'-monophosphate (AMP). AMP is further catabolized to adenosine and then inosine which then forms HX.[2,4,20,21] In 1977 Saugstad *et al.*[22] demonstrated that severe hypoxemia induced by reducing the inspired oxygen content or clamping the endotracheal tube in dogs produced a significant rise in venous and arterial plasma HX levels. In an intestinal hypoxia-ischemia model in cats, Schoenberg *et al.*[23] demonstrated a drastic rise in HX levels in intestinal mucosal tissue during 120 minutes of hypoperfusion. Hypoxanthine was shown to be a marker of hypoxia in newborns[24] as a significantly higher peak in plasma HX concentration was demonstrated in asphyxiated versus normal infants in the first 10 to 20 minutes after birth.

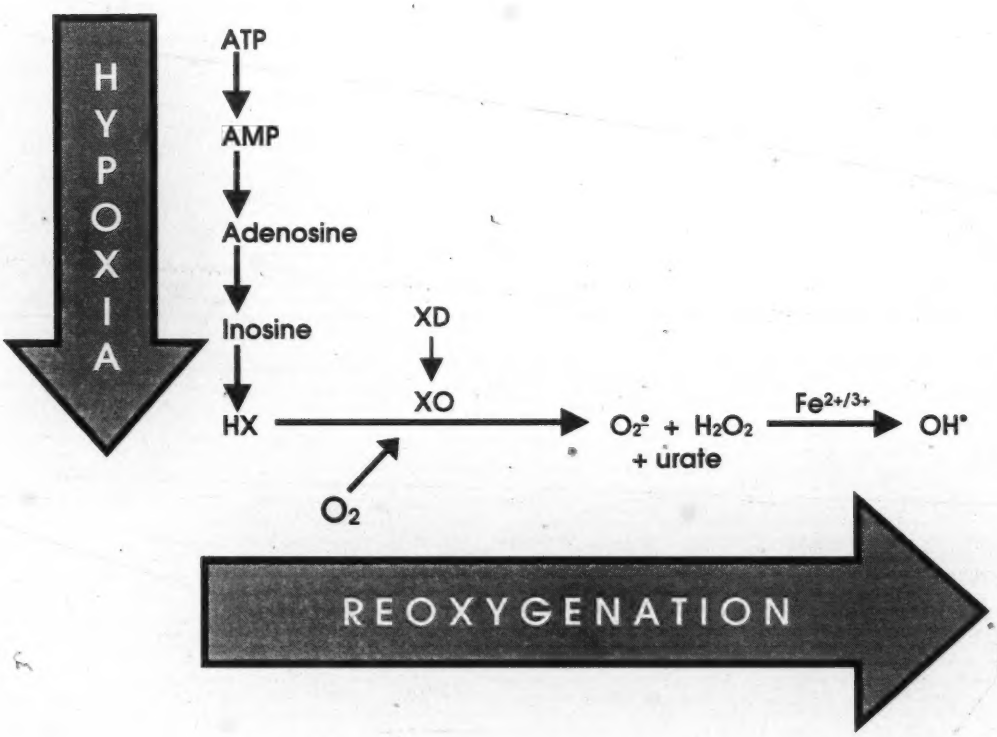
Xanthine oxidoreductase is a ubiquitous enzyme, most abundant in liver and small intestine.[11] It is a soluble, cytosolic protein existing in two forms: xanthine dehydrogenase (XD) and XO. In normal, non-ischemic cells xanthine oxidoreductase exists predominantly as the innocuous XD, whose

physiologic function is nucleic acid degradation.[25] Xanthine dehydrogenase utilizes oxidized nicotinamide adenine dinucleotide (NAD^+) as the electron acceptor in the oxidation of purines, forming xanthine.[20,26] During hypoxia, XD is converted to XO (D-to-O conversion) either irreversibly by proteolytic cleavage, or reversibly by oxidation of sulfhydryl residues on the enzyme.[26,27] In its XO form, the enzyme uses O_2 as an electron acceptor instead of NAD^+, and generates $O_2^{\bullet}$ and H_2O_2 by the oxidation of HX (see figure 3-3), which is abundant in hypoxic tissue.[20,26] The XO system has been recognized as the most important OFR-generating enzyme in hypoxia-reoxygenation and ischemia-reperfusion injury in many organs systems. Zweier *et al.* examined the mechanism of generation of OFRs in human[28] and porcine[29] vascular endothelial cells in response to hypoxia and reoxygenation. Xanthine oxidase and HX were shown to be present in these cells and were the primary source of OFRs. The reoxygenated human endothelial cells were shown to generate $O_2^{\bullet}$ radicals, which further reacted with iron in the Haber-Weiss reaction to form $OH^{\bullet}$ radicals, which in turn caused cell death.

In summary, the XO system has been recognized as the most important contributor to OFR-induced injury during hypoxia and reoxygenation. Hypoxia primes the cells for OFR generation by increasing HX, the substrate for the enzyme. Upon reoxygenation, there is an influx of the second substrate necessary for the XO pathway, O_2. Hypoxanthine is converted to xanthine and uric acid, thereby reducing O_2 to $O_2^{\bullet}$ and concomitantly generating H_2O_2, which then goes on to form $OH^{\bullet}$.

FIGURE 3-3

GENERATION OF OXYGEN FREE RADICALS BY THE XANTHINE OXIDASE-HYPOXANTHINE SYSTEM



Pathway for oxygen free radical generation by the xanthine oxidase-hypoxanthine (XO-HX) system. During hypoxia, ATP is broken down to HX, and xanthine dehydrogenase (XD) is converted to XO. When molecular oxygen (O_2) is reintroduced during reoxygenation, XO catalyzes the formation of the superoxide radical ($O_2^{\bullet}$) and hydrogen peroxide (H_2O_2), which then goes on to form the highly cytotoxic hydroxyl radical ($OH^{\bullet}$).

Nitric Oxide and Peroxynitrite

Nitric oxide (NO) is a ubiquitous cellular messenger with important vasoactive properties.[30,31] Nitric oxide itself is a free radical, as it contains an unpaired electron in its outer shell. However unlike the cytotoxic and highly reactive free radicals discussed thus far, NO is not very toxic at biological concentrations.[31] Due to its unpaired electron, NO does however react rapidly with other free radicals. The most well-documented reaction is the generation of the highly toxic peroxynitrite anion ($ONOO^-$),[30-35] as shown in figure 3-4. Nitric oxide and $O_2^{\bullet}$ react in a near-diffusion-limited rate, i.e. nearly every collision of NO and $O_2^{\bullet}$ results in the irreversible formation of $ONOO^-$. The formation of $ONOO^-$ is usually limited by the minute concentrations of $O_2^{\bullet}$ in cells, which are normally scavenged by SOD. However in the setting of hypoxia and reoxygenation, endothelial cells, macrophages and neutrophils generate a burst of $O_2^{\bullet}$, and at the same time, NO synthase is induced to produce increased quantities of NO from the same cells. In this setting, NO out-competes SOD in the reaction with $O_2^{\bullet}$, and $ONOO^-$ is formed.[31] Yasmin *et al.*[32] demonstrated the production of $ONOO^-$ in isolated rat hearts following 20 minutes of ischemia. Peroxynitrite production, measured in the coronary effluent as dityrosine, peaked at 30 seconds of reperfusion to 175% of baseline values. Production of $ONOO^-$ was inhibited by the NO synthase inhibitor N^G-monomethyl-*l*-arginine.

Peroxynitrite is itself not a free radical, but it is a powerful oxidant whose mechanisms of cytotoxicity include sulfhydryl oxidation, tyrosine nitration, lipid peroxidation, and impairment of mitochondrial respiration.

FIGURE 3-4

NITROGEN-DERIVED OXIDANTS AND OTHER REACTIONS WITH NITRIC OXIDE

Formation of peroxynitrite anion ($ONOO^-$) by the reaction between nitric oxide and superoxide

$$NO^{\bullet} + O_2^{\bullet} \longrightarrow ONOO^-$$

Nitrosothiol formation with glutathione

$$ONOO^- + GSH \longrightarrow GSNO + H_2O$$

Glutathione oxidation by peroxynitrite

$$ONOO^- + 2GSH \longrightarrow GSSG + NO_2 + H_2O$$

Nitration of tyrosine residue on protein

$$ONOO^- + \text{tyrosine-R} \longrightarrow \text{nitrotyrosine-R}$$

The most well-documented action of $ONOO^-$ is the oxidation of sulfhydryl bonds in proteins and non-protein thiols. The sulfhydryl bond is critical to the active site of many enzymes, it is important for maintaining the native conformation of many proteins, and it is a common target for free radicals.[33,36] Radi *et al.*[33] demonstrated that $ONOO^-$ reacts with sulfhydryl groups on bovine serum albumin or non-protein cysteine approximately 10^3 times faster than H_2O_2, indicating it is a more potent oxidant that H_2O_2.

Glutathione oxidation is an important mechanism for detoxification of $ONOO^-$.[31] Glutathione (GSH) is a tripeptide thiol (contains sulfhydryl bond) which reacts with $ONOO^-$ in the formation of GSSG or s-nitrosoglutathione (GSNO),[33,37] (see figure 3-4). Nitric oxide is a well know vasodilator, and is used clinically for the treatment of pulmonary hypertension in newborns and adults.[38] It has been suggested that nitrosothiols such as GSNO are responsible for the vasodilatory effect of NO.[31,39] Wu *et al.*[39] demonstrated that addition of $ONOO^-$ and GSH to isolated bovine pulmonary artery rings caused relaxation, which was attributed to the formation of GSNO. Unlike NO, which is rapidly scavenged by red blood cell oxy-hemoglobin, nitrosothiols are very stable in plasma with a half life of about 40 minutes.[31] Thus GSNO and other nitrosothiols may have prolonged vasodilator effects due to a release of NO over prolonged time periods.[31,39]

Nitrotyrosine is formed by the nitration of tyrosine residues on proteins by $ONOO^-$.[40] It is a very stable molecule and has been used as a marker for $ONOO^-$.[34,35] Nitrotyrosine can have important functional consequences to proteins. Addition of the bulky nitro group (—NO_2) onto tyrosine, which is

normally a hydrophobic amino acid, introduces a negative charge into nitrated proteins and can cause conformational changes to important proteins.[35] Tyrosine nitration has been reported in a number of different disease states in humans and experimental animals. Nitrotyrosine has been found in the lungs of infants with respiratory disease, in the plasma of infants with chronic lung disease, and in intestinal tissue in necrotizing enterocolitis.[41] It has also been documented in animal models of ischemia-reperfusion and hyperoxia.[41] In addition to tyrosine, $ONOO^-$ also targets guanine bases in DNA, forming nitroguanine which could be used as a marker of $ONOO^-$-induced DNA damage.[42]

Neutrophil Peroxidases

Neutrophils have been demonstrated as mediators of hypoxia-reoxygenation injury in a variety of organs, such as the intestine, lungs, heart, kidneys, brain, liver, and muscles.[14,43-46] Some of the earliest evidence of neutrophil involvement in hypoxia-reoxygenation injury was in experimental neutrophil-deplete reoxygenation. In 1987, Hernandez *et al.*[47] examined microvascular permeability in cat intestine following partial ischemia and reperfusion, and showed that animals rendered neutropenic by pre-treatment with anti-neutrophil serum had significantly attenuated microvascular injury. Brown *et al.*[48] demonstrated that reoxygenation of ischemic rat intestine with oxygenated perfluorochemical yielded minimal damage, compared to reoxygenation with perfluorochemical containing

harvested leukocytes, or reoxygenation with blood, both of which caused significant histologic damage to the intestine. Kraemer *et al.*[49] determined the role of neutrophils in hypoxia-reoxygenation-induced cardiac dysfunction in the isolated buffer-perfused rabbit heart. Hearts perfused with a neutrophil-free buffer during reoxygenation after 20 minutes of hypoxia had a significantly faster recovery of contractility compared to hearts perfused with buffer containing neutrophils. These studies support the concept that neutrophils contribute to organ injury and dysfunction during hypoxia-reoxygenation.

Neutrophil activation and infiltration occurs during reoxygenation of hypoxic tissue.[1,14,50] Oxygen free radicals play an important role in the recruitment of neutrophils to post-hypoxic tissue, and it is hypothesized that XO-derived OFRs initiate the production and release of proinflammatory agents, with subsequent attraction and activation of neutrophils.[14,20,50,51] Ichikawa *et al.*[52] demonstrated that human vein endothelial cells exposed to anoxia and reoxygenation developed adhesions with neutrophils. Neutrophil-endothelial adhesion was inhibited by oxypurinol, a potent inhibitor of XO, as well as catalase, which catalyzes the detoxification of H_2O_2 to H_2O, suggesting that both XO and H_2O_2 play a role in neutrophil adhesion to the endothelium during hypoxia-reoxygenation.

Once activated neutrophils adhere to the microvascular endothelium, they migrate into the surrounding tissue, and release proteolytic enzymes, including elastase, collagenases, and proteases, which damage the endothelial basement membrane and extracellular matrix.[20,50]

The activated neutrophil is an important source of OFRs during reoxygenation, generating $O_2^{\bullet}$, H_2O_2, and hypochlorous acid (HOCl) via the NADPH oxidase and myeloperoxidase enzymes,[14,45,50] (see figure 3-5). Myeloperoxidase (MPO) is a membrane-bound enzyme that catalyzes the conversion of H_2O_2 to HOCl, a powerful oxidant and chlorinating agent. Myeloperoxidase is found almost exclusively in neutrophils, accounting for up to 5% of neutrophil dry weight, and is well-established as a sensitive marker of neutrophil infiltration and OFR production.[14,44] Cuzzocrea *et al.*[53] demonstrated a 3-fold rise in MPO activity following splanchnic artery occlusion and reperfusion in rats. Grisham *et al.*[46] demonstrated an 18-fold increase in tissue MPO activity after reperfusion of ischemic cat intestines. The influx and activation of neutrophils was attenuated by pretreatment with the XO-inhibitor allopurinol or the OFR-scavenger SOD, supporting the theory that XO-derived OFRs are responsible for neutrophil infiltration and activation during hypoxia-reoxygenation.

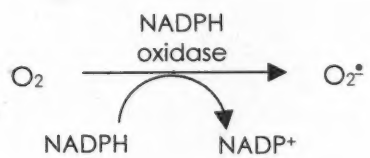
In summary, OFRs lead to neutrophil activation and infiltatration, which is the source of further OFR generation. Neutrophil MPO activity is an important and sensitive marker of OFR generation by activated neutrophils.

FIGURE 3-5

NEUTROPHIL FREE RADICAL AND OXIDANT GENERATION

Formation of hypochlorous acid (HOCl) in neutrophils, catalyzed by myeloperoxidase (MPO)

Superoxide ($O_2^{\bullet-}$) formation by neutrophil NADPH oxidase



Mitochondrial generation of free radicals

Under physiologic conditions, oxygen is tetravalently reduced in the mitochondrial electron transport chain to water.[10-12] However studies in isolated mitochondria have shown that oxygen can undergo one-electron reduction in the electron transport chain, yielding $O_2^{\bullet}$.[54-56] It is estimated that only 1-2% of oxygen utilized by mitochondria forms $O_2^{\bullet}$, and these physiologically-generated OFRs are inactivated by the endogenous scavenger mechanisms available within the cells.[56]

In 1993, Ambrosio *et al.*[56] were the first to demonstrate that the mitochondrial electron transport chain is also a source of pathologic OFR generation during reoxygenation, in a model of an isolated, perfused rabbit heart subjected to 30 minutes of global ischemia followed by reoxygenation with leukocyte-free buffer. They observed an increase in OFR generation and OFR-induced lipid peroxidation upon reoxygenation, which was significantly reduced in hearts treated with sodium amobarbital, an inhibitor of mitochondrial respiration at the NADH dehydrogenase site. They also observed an improvement in recovery of left ventricular function in the sodium amobarbital treated hearts during reoxygenation. They concluded that mitochondrial respiratory chain electrons were indeed an important source of oxygen radical generation during reoxygenation. Radi *et al.*[57] demonstrated that exposure of rat heart mitochondria to $ONOO^-$ caused not only an inhibition of normal mitochondrial respiration, but also an increase in mitochondrial H_2O_2 generation. They hypothesized that inhibition of the

mitochondrial electron transport chain allowed greater electron leakage to O_2, with the resultant formation of $O_2^{\bullet}$, which then rapidly dismutates to H_2O_2. Thus, mitochondrial OFR generation is important not only under physiologic conditions, but also in the setting of hypoxia-reoxygenation.

Cellular Mechanisms of Free Radical Injury

Whether originating from XO, neutrophils, NO, or mitochondrial respiration, OFRs ultimately cause reoxygenation injury by common mechanisms. The main cellular targets of OFRs are (1) protein sulfhydryl groups, (2) lipid membranes, (3) mitochondria, and (4) DNA.

Sulfhydryl bond oxidation by OFRs and oxidants can cause cellular damage by inactivating essential enzymes, membrane transport proteins, or structural proteins, thereby compromising the cell's ability to function and maintain homeostasis.[1,14,58]

Polyunsaturated fatty acids are present in high concentrations in cell membranes, and are very susceptible to OFR attack. Plasma membranes, nuclear membranes, and other organelles are targets of lipid peroxidation.[1,14,58] Studies have shown that XO-derived OFRs, neutrophil-derived OFRs, as well as $ONOO^-$, induce membrane lipid peroxidation,[59,60] with the resultant formation of lipid hydroperoxides (LOOH).[14] Lipid peroxidation can lead to cell lysis and death, either directly, or via LOOH which change membrane fluidity and permeability.[1,14] Lipid peroxidation of neutrophil membranes also contributes to the release of proteases and peroxidases.[1]

Oxygen free radicals are known to target mitochodria.[56-58] Peroxynitrite and OFRs impair mitochondrial function, by inhibiting the respiratory chain. Structurally mitochondria are targeted as well, with lipid peroxidation of mitochondrial membranes, and resultant mitochondrial swelling. This will have important consequences to the cell itself, as mitochondrial dysfunction will lead to inadequate aerobic metabolism and ATP generation necessary for cellular function, homeostasis, and survival.[57]

Finally, DNA strand breakage can occur with the reaction of nucleic acids and OFRs.[1,14,58] Neutrophil-generated HOCl can cause the chlorination of purine bases,[14,20] and nitration of guanine bases can occur by the reaction with $ONOO^-$.[42]

Effects of oxygen free radicals on specific organ systems in the newborn

It has been hypothesized that OFRs are responsible for a number of specific "oxygen free radical diseases of the newborn", including chronic lung disease, necrotizing enterocolitis, retinopathy of prematurity, periventricular leukomalacia and patent ductus arteriosus.[5,6] Indeed, OFRs have been implicated as mediators of hypoxia-reoxygenation and ischemia-reperfusion injury in many organ systems, including the heart,[58,61-65] bowel,[18,46,66-70] lung,[71-75] brain,[76-78] and kidneys,[79] as discussed in greater deal in chapters 5, 6, 7, 8, and 9, respectively.

Defense strategies against oxygen free radicals in newborns

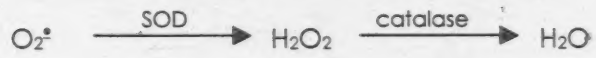
Cells are equipped with endogenous mechanisms to counteract the detrimental effects of OFRs. Three of the most important enzymatic antioxidant mechanisms are SOD, catalase, and glutathione peroxidase (GSH-Px).[10,14,71] Superoxide dismutase is a highly specific enzyme which catalyzes the dismutation of $O_2^{\bullet}$ to H_2O_2. Catalase and GSH-Px then detoxify H_2O_2 to water. This stepwise detoxification prevents the formation of the highly toxic $OH^{\bullet}$ radical (see figure 3-6).[10,14,71]

The GSH antioxidant system is the major antioxidant mechanism in the cell, maintaining enzymes and other cellular components in a reduced state.[80-83] Glutathione is a tripeptide synthesized within cells from the amino acids glutamate, cysteine and glycine, catalyzed by GSH synthetase in an ATP-dependent reaction.[82] Increased cellular levels of cysteine, for example from the exogenous administration of N-acetylcysteine, lead to an increase in cellular GSH, as cysteine is the limiting substrate for GSH synthesis.[82] Glutathione functions as an antioxidant during periods of oxidative stress, by reacting directly with OFRs and oxidant molecules. This reaction is catalyzed by the antioxidant enzyme GSH-Px, with the resultant formation of the oxidized form of GSH, glutathione disulfide (GSSG).[82] Glutathione disulfide is then reduced back to GSH by GSH reductase, accompanied by the oxidation of NADPH.

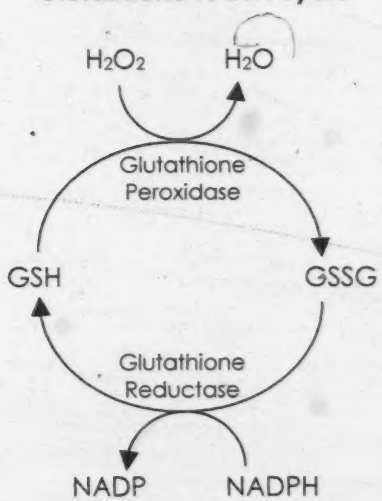
FIGURE 3-6

ENDOGENOUS CELLULAR DEFENCES AGAINST FREE RADICALS

Superoxide dismutase and catalase



Glutathione redox cycle



Other glutathione reactions

$$GSH + R\cdot \longrightarrow GSSG + RH$$

$$GSH + LOOH \longrightarrow GSSG + LOH$$

$$GSH + ONOO^- \longrightarrow GSNO + H_2O$$

Superoxide dismutase (SOD) catalyzes the dismutation of the superoxide radical ($O_2^{\bullet}$) to hydrogen peroxide (H_2O_2), which is then detoxified by either catalase or glutathione peroxidase. Glutathione (GSH) is the major antioxidant in most cells, and reduces oxygen and nitrogen free radicals and oxidized proteins and lipids, yeilding glutathione disulfide (GSSG), the oxidized form of glutathione. ($R\cdot$ = free radical, LOOH = lipid hydroperoxide, LOH = lipid alcohol, $ONOO^-$ = peroxynitrite anion, GSNO = s-nitrosoglutathione)

Under normal conditions, most GSH in cells exists in the reduced, thiol (sulfhydryl) form.[82] An increase in GSSG has been used as a marker for increased oxidant stress, or a decrease in antioxidant capacity.[81,84,85] De la Asuncion *et al.*[84] showed an increase in oxidized-to-reduced glutathione ratio (GSSG:GSH) with age in liver and brain of rats and mice, which strongly correlated with degree of mitochondrial DNA damage. They furthermore showed that animals treated with exogenous antioxidants vitamin E and C had lower GSSG:GSH ratio as well as less DNA damage. Carbonell *et al.*[83] demonstrated that depletion of glutathione by injecting phorone caused an increase in lipopolysaccharide-induced oxidative damage in rats, as measured by an increase in lipid peroxidation and a decrease in total antioxidant capacity in lipopolysaccharide+phorone treated rats compared to lipopolysaccharide alone. Cheung *et al.*[80] demonstrated in isolated rat hearts subjected to either ischemia-reperfusion, or $ONOO^-$ infusion, that administration of high concentrations of GSH attenuated mechanical dysfunction in a concentration-dependent fashion. Furthermore the concentration of dityrosine, a marker of $ONOO^-$ formation, in the coronary effluent was reduced in the GSH-treated hearts. They concluded that GSH plays an important role in the detoxification of nitrogen oxidants as well as OFRs.

Under physiologic conditions, endogenous cellular antioxidants are sufficient to detoxify the small amount of OFRs that are generated during normal mitochondrial oxidative phosphorylation. However, situations with increased oxidative stress and OFR formation, such as perinatal asphyxia and

resuscitation, may overwhelm the intrinsic protective antioxidant systems and result in cellular and tissue injury. It has been suggested that neonates have reduced cellular oxidative defences due to a prematurity of antioxidant systems,[5] particularly in premature infants.[86] Lindeman *et al.*[87] studied plasma concentrations of various antioxidants in infants and adults. They showed similar levels of antioxidants in term and preterm infants, but infants had lower levels of vitamin E, sulfhydryl groups (including GSH), and beta-carotene than adults, and higher levels of vitamin C compared to adults. Serum total antioxidant activity was measured in neonates by Sullivan and Newton,[88] and increased serum antioxidant activity was found with increasing birth weight. Furthermore, newborn infants were found to have a significantly lower serum antioxidant activity compared to adults.

McElroy *et al.*[89] studied SOD, catalase, and GSH-Px levels during human development, and showed that pulmonary GSH-Px and SOD levels remain unchanged from early gestation to 3 months following birth, however lung catalase concentration increased during gestation. Fryer *et al.*[90] also showed no gestational changes in lung GSH-Px in humans. Strange *et al.*[91] demonstrated that lung SOD did not increase during gestation in humans, but erythrocyte SOD did increase.

McElroy *et al.*[89] also showed that hepatic SOD and catalase increased from early gestation to 3 months after birth in humans, although liver GSH-Px remained relatively constant. In newborn rats, lower levels of hepatic GSH has been documented compared to adult rats.[81]

Intestinal antioxidant enzymes have been studied in piglets,

demonstrating an increase in intestinal SOD and a decreased GSH and GSH-Px in 1- and 3-day old piglets compared to 2-week and 1-month old piglets.[92] In this study, intestinal catalase was undetectable.

Upon exposure to oxidant stress, antioxidants have been shown to increase in neonates. Rosenfeld *et al.*[93] demonstrated that plasma SOD levels increased in newborns exposed to an oxygen challenge. Vento *et al.*[85] demonstrated an increase in GSSG, GSH-Px, SOD and catalase at birth or 72 hours after birth in asphyxiated newborns compared to controls.

In summary, newborns have lower levels of some key cellular antioxidants compared to adults in various organs and in plasma. Newborns appear able to increase the level of antioxidant enzymes in response to oxidative stress. Further research is necessary to fully understand the essential differences between neonates and adult in their capacity to counteract oxidative stress.

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CHAPTER 4

The Experimental Model: Acute Hypoxia and Graded Reoxygenation in Newborn Piglets

Use of a Newborn Piglet Model for Perinatal Asphyxia

The newborn piglet has been utilized extensively in perinatal research, and has been described as the best physiologic model among common laboratory species for comparison to human infants.[1,2] Pigs are anatomically and physiologically similar to humans, and the degree of maturity at birth is similar to human infants.[1] Pigs have been found to be tolerant to physiologic stress, surgical manipulation, and administration of anesthetic agents, thus are an ideal surgical and physiologic model of severe perinatal stress.[3]

Pigs are the experimental animal of choice in cardiovascular research due to similarities in size and distribution of blood vessels, blood pressure, heart rate, cardiac output, ventricular function, and regional organ blood flow.[4,5] The postnatal development of cardiovascular regulation in newborn piglets has been studied, and it has been shown that they have similar cardiovascular reflex responses including vagal activity, baroreceptor function, and neural regulation of mesenteric flow and vascular resistance. They also have similar cardiovascular responses to human neonates to stresses such as hypoxia, hypercapnia and hemorrhage, common stresses in the neonatal period.[6]

The newborn piglet pulmonary vasculature has been studied, and found to be similar structurally to that of the human at the time of birth, and it is remodelled in a similar fashion in the postnatal period, although growth is more rapid than in humans. Pulmonary hemodynamics at rest and with anesthesia are similar in piglets and humans, and piglets develop potent

pulmonary vasoconstriction in response to alveolar hypoxia in a characteristic fashion, as seen in human newborns.[7]

The piglet has been used as an ideal animal model of necrotizing enterocolitis, as their gastrointestinal tracts are similar physiologically and anatomically to humans.[1,8] The mesenteric vascular response to cardiogenic shock has been studied in pigs, and is similar to humans. Importantly, in the face of severe acute intestinal hypoperfusion the systemic circulation in pigs is less susceptible to bacteremia, unlike in dogs where secondary sepsis is more likely to occur as a result of bacterial translocation.[3] This will prevent hemodynamic alterations due to sepsis, when studying a model of perinatal asphyxia where cardiogenic shock is the more relevant component, as in humans.

Pigs have a similar renal structure as humans, and they also share similar renal physiology and fetal and neonatal development as in humans.[9] Pigs have the same range of normal serum chemistry and hematology values as in humans, as well as acid base balance and blood gas values.[10-12]

In summary, extensive research in pigs over many decades has established this species as one of the closest to human, ideal for perinatal, cardiovascular, pulmonary, and gastrointestinal studies.

Use of an Intact Animal Model

In the study of perinatal asphyxia and the effects of hypoxia and reoxygenation, important physiologic changes in the whole organism and

interactions between the various organ systems can only be evaluated with an intact animal model. Indeed, the sympatho-adrenal vascular responses to hypoxia and the systemic vascular effects and distant organ effects of oxygen free radicals (OFRs) can only be appreciated when these organ systems are able to interact. Furthermore, an intact animal model is more applicable to the clinical scenario.

Use of an Acute Instrumentation Model

Oxygen free radical activity has been shown to be maximal in the minutes to hours following reoxygenation or reperfusion,[13,14] thus measurement of tissue indices of oxidative stress and OFR-related injury should be carried out shortly following reoxygenation. In our protocol, reoxygenation proceeded for four hours, which allowed us to document the hemodynamic changes during reoxygenation, as well as obtain tissue for analysis at an appropriate timepoint for detection of OFR-related injury. The main disadvantage of an acute model is the physiologic stress of acute surgical intervention, which was minimized by limiting the time of the surgical procedure (less than 75 minutes), and allowing a period of stabilization until hemodynamic and blood flow parameters were stable (±10% change over 20 minutes). Furthermore, a sham-operated group of piglets served as a control for the acute effects of surgical intervention and anesthesia.

The Hypoxia-Reoxygenation Model

The protocol of hypoxia-reoxygenation used in these studies consisted of hypoxemia (P_aO_2 30-40 mmHg) for 2 hours duration followed by reoxygenation with 21%, 50% or 100% oxygen for 1 hour then 21% oxygen for the remaining 3 hours. The duration of hypoxemia has been previously established,[15] and allowed for clinically relevant changes in hemodynamics and acid-base status, as observed in newborns with severe perinatal asphyxia. The current protocol allowed us to control the confounding variables during hypoxia, including severity and duration of hypoxia and hemodynamic and biochemical sequelae, in order to focus on the differences during reoxygenation. To study the concentration-dependent effects of oxygen, piglets were reoxygenated for 1 hour with different oxygen concentrations, followed by 3 hours with 21% oxygen, which is comparable to the clinical scenario of newborn resuscitation, whereby initial management consists of administration of 100% oxygen, and inspired oxygen is later decreased when the newborn is stabilized and transferred to a neonatal intensive care unit.

Use of Transonic Flow Probes for Blood Flow Measurement

The use of ultrasonic transit time flow probes for measurement of blood flow volume has been well-validated and shown to be accurate and reliable.[16] Transonic™ flow probes have been used extensively for animal research, including carotid, mesenteric and renal arterial flow measurements

and cardiac output. Ultrasonic flow probes allow for direct and continuous measurement of blood flow, with a reported variability of approximately ±10%. In our surgical protocol all attempts were made to minimize this variability by following a consistent surgical approach with the placement of flow probes, limiting the surgical dissection around the arteries, and ensuring correct alignment of flow probes. The radiolabeled or fluorescent microsphere techniques for blood flow determination are similarly accurate, and have the advantage of being non-invasive, however do not allow for continuous blood flow measurements.[17]

The Choice of Drugs for Anesthesia and Analgesia

Anesthesia has been studied extensively in pigs, and they are tolerant to both inhalant anesthetics such as halothane and intravenous agents such as benzodiazepines and narcotics.[18-20] Halothane causes important cardiovascular effects in both humans and pigs when given at surgically relevant concentrations (about 1% end-tidal concentration, minimal alveolar concentration 1.5),[19] so the use of halothane was limited to the first 10 to 15 minutes of surgical instrumentation in our model. Midazolam, fentanyl and pancuronium are medications commonly used in critically ill newborns, and cause minimal hemodynamic disturbances in piglets. The administration of these medications by continuous intravenous infusion further reduces the already mild hemodynamic effects. Acepromazine, similar to chlorpromazine used in humans, is one of the most commonly used sedatives in veterinary medicine, as it has proven to be safe and without significant side effects.[21]

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CHAPTER 5

Myocardial Glutathione Correlates with Cardiac Function in Hypoxemic Newborn Piglets Reoxygenated with 21%, 50% or 100% Oxygen

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INTRODUCTION

Oxygen free radicals (OFRs) have been established as the primary mediators of myocardial dysfunction and damage following ischemia-reperfusion[1-4] and hypoxia-reoxygenation.[5-7] Studies have shown that the newborn myocardium is more resistant to hypoxic stress[8-10] and free radical-mediated reperfusion injury[11-13] than the adult myocardium, however newborns with severe hypoxemia will usually manifest immediate cardiovascular changes including hypotension and bradycardia.[14,15] Evidence of myocardial injury including elevated creatine kinase MB isoenzyme (CK-MB), electrocardiographic evidence of ischemia, clinical manifestations of ventricular dysfunction or atrio-ventricular regurgitation, or significant myocardial necrosis causing death, has been reported in 21-75% of infants with severe perinatal asphyxia.[15-18]

While hypoxia and ischemia can result in impaired ventricular contractility,[14] subsequent reperfusion or reoxygenation of hypoxic-ischemic myocardium can lead to additional impairment of cardiac function, termed myocardial stunning.[19] There is direct evidence that post-ischemic reperfusion is associated with a burst of OFR formation, which is absent when isolated hearts are reperfused with anoxic buffer,[20] and that the magnitude of free radical generation is proportional to the severity of ischemia.[21] Free radicals have been directly implicated in causing impaired contractile function and myocardial stunning in the minutes to hours following reperfusion.[4,21] Furthermore, exposure of the non-ischemia-reperfused myocardium to OFRs has been shown to produce extensive myocardial

necrosis, which is attenuated by the administration of OFR scavengers.[22]

Myocardial cells contain endogenous antioxidants which act as defence mechanisms against the detrimental effects of OFRs.[5,23] Glutathione (GSH) is an important cellular antioxidant which can detoxify OFRs, with the resultant formation of oxidized glutathione (GSSG).[24] Studies have shown that myocardial ischemia-reperfusion causes a depletion of GSH levels and an increase in GSSG,[25,26] and that GSH depletion correlates with the extent of myocardial cell death.[27] Furthermore exogenous GSH administration[28] or stimulation of the GSH redox cycle[27] has been shown to decrease OFR generation and attenuate ischemia-reperfusion-induced contractile dysfunction.

Previous human studies have shown that 21% oxygen is as effective as 100% in the cardiovascular recovery from severe perinatal asphyxia,[29,30] and that resuscitation with higher oxygen concentrations resulted in prolonged oxidative stress, evidenced by elevated GSSG levels and increased glutathione redox ratio (GSSG:GSH) in the blood.[31] In hypoxic newborn piglets resuscitated with 21% or 100% oxygen, Rootwelt *et al.* demonstrated no difference in myocardial blood flow,[32] cardiac output, or mean arterial pressure,[33] between the two groups, and Medbo *et al.*[34] observed an increase in cardiac output at 5 minutes of reoxygenation with 21% oxygen, and no other differences in systemic or pulmonary hemodynamics. To our knowledge, antioxidant status and assessment of histologic reoxygenation injury has not been previously compared in newborn animals resuscitated with 21%, 50% or 100% oxygen. We hypothesized that higher concentrations

of oxygen used during resuscitation would result in cardiomyocyte injury and GSH depletion, which in turn would impair myocardial function.

The objective of the present study was to determine, in an intact newborn animal model, whether myocardial function and injury are affected by the concentration of oxygen used in resuscitation following severe hypoxemia, and whether cardiovascular changes correlate with myocardial GSH status.

METHODS

Animals. All experiments were conducted in accordance with the guidelines and approval of the Health Sciences Animal Policy and Welfare Committee of the University of Alberta. Thirty-two newborn Yorkshire-Landrace piglets 1-3 days of age weighing 1.5-2.1 kg were obtained on the day of experimentation from a local farm.

Surgical procedure. Anesthesia was induced with inhaled halothane at 5% which was then decreased to 2-3%. A 5-French Argyle™ single lumen arterial catheter (Sherwood Medical Co., St. Louis, MO) was inserted into the distal aorta via the right femoral artery, and was attached to a pressure transducer and monitor (Hewlett Packard 78833B monitor, Hewlett Packard Co., Palo Alto, CA) for continuous systemic arterial blood pressure and heart rate (HR) measurements. A 5-French Argyle™ double lumen catheter was inserted to the level of the right atrium via the right femoral vein for central venous pressure (CVP) measurement and for the administration of medications and fluids. A tracheotomy and endotracheal intubation was performed, and pressure-controlled assisted ventilation was commenced (Sechrist infant ventilator model IV-100, Sechrist Industries Inc., Anaheim, CA) with pressures of 19/4 cm H_2O at a rate of 18-20 breaths/minute. Oxygen saturation was measured with a pulse oximeter (Nellcor, Hayward, CA), and inspired oxygen concentration (F_IO_2) measured by an Ohmeda 5100 oxygen monitor (Ohmeda Medical, Laurel, MD), was maintained at 0.21-0.24 for oxygen saturations between 90% and 100%. Halothane was then discontinued and anesthesia was maintained with intravenous fentanyl 5-10

μg/kg/h, midazolam 0.1-0.2 mg/kg/h and pancuronium 0.05-0.1 mg/kg/h, with additional boluses as necessary, and acepromazine 0.25 mg/kg. A left anterior thoracotomy in the third intercostal space was performed and a six-millimetre transit time ultrasound flow probe (6SB906, Transonic Systems Inc., Ithica, NY) was placed around the main pulmonary artery and attached to a Transonic T206 2-channel small animal blood flow meter to continuously measure cardiac output. A 20-gauge Arrow™ catheter (Arrow International, Reading, PA) was inserted into the main pulmonary artery and attached to a pressure transducer to continuously measure pulmonary arterial pressure. All incisions were closed or covered to minimize evaporative heat loss. Maintenance fluids during experimentation consisted of 5% dextrose in water at 7.5 mL/kg/h and 0.9% NaCl at 2.5 mL/kg/h. Piglet temperature was maintained at 38.5-39.5°C using an overhead radiant warmer and a heating pad.

Piglets recovered from the surgical procedure until baseline hemodynamic measures were stable (change less than ±10% over 20 minutes), with pH 7.35-7.45 and P_aCO_2 30-45 mmHg. Mean systemic arterial blood pressure (MAP), HR, cardiac output, CVP, mean pulmonary arterial pressure (PAP), and oxygen saturation were continuously monitored and recorded throughout the experiment. Analogue outputs of the pressure amplifiers and flow monitor were digitized by a DT 2801-A analogue to digital converter board (Data translation, Ontario, Canada) in a Dell 425E personal computer. Software was custom written using the Asyst programming environment.

Experimental protocol. Piglets were block randomized into four groups (n=8 each). In three groups, hypoxemia was induced by ventilating the piglets with an F_iO_2 of 0.10 by increasing the concentration of inhaled nitrogen gas. The F_iO_2 was adjusted as tolerated by the piglet, to obtain severe hypoxemia (P_aO_2 of 20-40 mmHg) for 2 hours. Following hypoxia, piglets were resuscitated with an F_iO_2 of 0.21 (21% group), 0.50 (50% group), or 1.0 (100% group) for 1 hour, followed by 0.21 for 3 hours. A control group of piglets was ventilated and oxygenated at an F_iO_2 of 0.21 for the 6 hours of study, with no period of hypoxia or reoxygenation. At the end of the experiment, piglets were euthanized with a bolus of 100 mg/kg pentobarbital.

Hemodynamic measures. Hemodynamic and regional blood flow recordings for data analysis were carried out at specified timepoints: baseline (0 minutes), 60 and 120 minutes of hypoxia, and 5, 15, 30, 60, and 240 minutes of reoxygenation, and calculated as a mean over two minutes of recording. Hemodynamic variables were calculated as shown in Appendix 1 (page 177).

Blood and tissue collection. Arterial blood samples were drawn and immediately processed for gas and acid-base analysis and hemoglobin measurements (Stat Profile, Nova Biomedical, Waltham, MA) at baseline, 30 and 120 minutes of hypoxia, and 30 and 240 minutes of reoxygenation. The measured hemoglobin values did not change significantly during the entire experiment. Immediately after the piglets were euthanized, the heart was removed and a portion of the left ventricle wall was snap-frozen in liquid nitrogen and stored at –80°C until biochemical analysis, while another sample

of the left ventricle was fixed in 10% formalin for histologic analysis.

Determination of left ventricular myocardial glutathione. Levels of total and oxidized GSH in the left ventricular myocardium were measured using a GSH assay kit (catalog no. 703002, Cayman Chemical, Ann Arbor, MI). Briefly, frozen samples of heart tissue were homogenized with 1 mL/100 mg of buffer containing 0.2 M 2-N-morpholino ethanesulphonic acid, 50 mM phosphate, and 1 mM EDTA, pH 6.0. Homogenates were centrifuged at 10 000 g for 15 minutes at 4°C, and the supernatant was collected and deproteinated with 10% metaphosphoric acid and 4 M triethanolamine, to avoid interference from sulfhydryl groups on proteins in the sample. A colorimetric microplate assay was performed by adding GSH reductase, $NADP^+$, and 5,5'-dithiobis-2-nitrobenzoic acid to the sample. The absorbance was measured after 25 minutes at 405 nm with a microplate reader (Titertek Multiskan Plus, MK II Type 314, Titertek, Huntsville, AL), and the total GSH concentration was calculated from a standard curve. To measure GSSG, deproteinated samples were incubated at room temperature for one hour with 1 M 2-vinylpyridine to completely derivatize the reduced GSH in the sample, and the colorimetric assay was carried out as above. Reduced GSH was calculated by subtracting GSSG from total GSH, and GSH redox status (GSSG:GSH) was obtained by calculating the ratio of oxidized-to-reduced GSH.

Histologic assessment and grading. Heart specimens preserved in formalin were prepared for routine histologic evaluation using hematoxylin and eosin staining. Sections were analyzed by two independent pathologists who were not aware of the study groups, and pathologic injury was

evaluated based on previously established criteria.[35]

Statistical analysis. Results are expressed as mean ± standard error of the mean. The hemodynamic variables were analyzed by two-way repeated measures (RM) analysis of variance (ANOVA) followed by one-way RM ANOVA or one-way ANOVA to determine differences within groups over time or between groups at each timepoint, respectively (SPSS 11.0.1, SPSS Inc., Chicago, IL, and Sigma Stat 2.0, Jandel Corp., San Rafael, CA). ANOVA was carried out as above on ranks if tests of normality or equal variances failed. Post-hoc testing using the Tukey method for one-way ANOVA and RM ANOVA or Dunnett's method for ANOVA on ranks was used for pairwise comparisons. One-way ANOVA with post-hoc LSD testing was used to determine differences in myocardial glutathione results. Pearson correlation analysis was used to assess the relationship between hemodynamic variables and GSH levels. Median histologic grade of injury was compared with the Kruskal-Wallis test, followed by the Mann-Whitney U test for pairwise comparison. Significance was defined as $p<0.05$.

RESULTS

Acid-base and blood gas status. Severe asphyxia was achieved in the piglets after exposure to alveolar hypoxia for 2 hours, with pH decreasing to 7.06 ± 0.04 and P_aO_2 decreasing to 32 ± 3 to 34 ± 2 mmHg in hypoxic groups compared to 7.44 ± 0.01 and 74 ± 5 mmHg in controls ($p<0.001$), respectively. Upon reoxygenation, P_aO_2 increased to 367 ± 15 mmHg in the 100% group ($p<0.001$ vs. all other groups), and P_aO_2 in the 50 % group (189 ± 14mmHg) was greater than in the 21% group (69 ± 5 mmHg) or controls (86 ± 10 mmHg) ($p<0.001$). P_aCO_2 remained similar to baseline and control values during the entire study period, and at the end of the study period, there were no significant differences in pH, P_aO_2, P_aCO_2, or bicarbonate among the four groups (data not shown).

Cardiac index. Severe hypoxemia for 2 hours resulted in a 66% ± 2% decrease in cardiac index, as shown in figure 5-1. Upon reoxygenation, cardiac index increased to a maximum of 180% ± 14% of control values in the 21% group ($p<0.005$) during the first 30 minutes of reoxygenation, while cardiac index in the 50% and 100% groups increased to 146% ± 20% and 155% ± 14% of controls, respectively, which was not significantly different from controls. In the control group, cardiac index decreased slightly at the end of the experiment compared to baseline values ($p<0.05$).

Heart rate. Hypoxemia initially caused tachycardia ($p<0.05$ vs. baseline) which subsequently resolved as shown in figure 5-2. With reoxygenation, HR increased in the 21% and 50% groups at 5 and 30 minutes

of reoxygenation, respectively, and remained elevated throughout the remainder of reoxygenation ($p<0.05$ vs. baseline). Heart rate in the 100% group was not different from baseline during reoxygenation. There were no significant differences in HR between experimental and control groups at any timepoint.

Stroke volume index. Two hours of hypoxemia resulted in a 64% ± 2% decrease in SVI, as shown in figure 5-3. During early reoxygenation, SVI transiently increased to 151 to 155% of control values, although this increase was not significant. Despite a decrease in SVI in controls at the end of the experiment compared to baseline values, SVI was not different between experimental and control groups during reoxygenation.

Mean arterial pressure and systemic vascular resistance index. Mean arterial pressure decreased by 61% ± 2% after 2 hours of hypoxia as shown in figure 5-4. MAP recovered immediately in all groups upon reoxygenation, but later decreased slightly in the 50% group compared to controls ($p<0.05$ at 30 to 60 minutes of reoxygenation). During the last 3 hours of experimentation, MAP decreased slightly in all experimental groups, including controls, compared to baseline values ($p<0.05$). Piglets subjected to hypoxia showed a decrease in SVRI during early hypoxia compared to controls ($p<0.05$) as shown in figure 5-5. Upon reoxygenation, there was a trend towards a decreased SVRI compared to controls in all groups, however this decrease was only significant in the 21% group during the first 30 minutes of reoxygenation. Systemic vascular resistance did not change in the control group compared to baseline values.

Pulmonary arterial pressure and pulmonary vascular resistance index. Hypoxia-induced pulmonary hypertension was observed during the first 90 minutes of hypoxemia, as shown in figure 5-6. Upon reoxygenation, PAP immediately and transiently increased to 194%, 138% and 146% of control values in the 21%, 50% and 100% groups, respectively ($p<0.05$), then recovered to control values by 60 minutes of reoxygenation in the 21% group and by 15 minutes in the 50% and 100% groups. Pulmonary artery pressure did not change over time in the control group compared to baseline values. Pulmonary vascular resistance increased significantly with hypoxemia in all groups compared to controls ($p<0.001$) (figure 5-7). Upon reoxygenation, PVRI recovered to control values immediately in all experimental groups, and remained similar to controls until the end of experimentation.

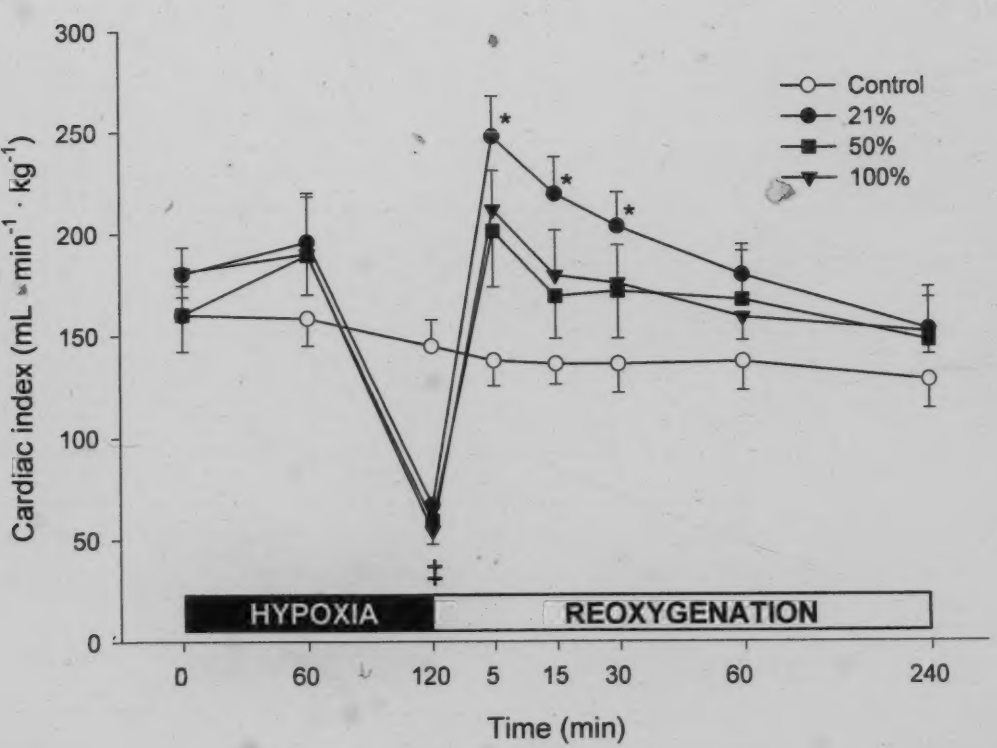
Left ventricular glutathione content. Levels of total GSH in left ventricular myocardium decreased after hypoxia and reoxygenation, as shown in figure 5-8(A), with a significant decrease in the 100% group ($p<0.05$ vs. control). The GSH redox ratio is shown in figure 5-8(B), and there is a modest increase in the GSSG:GSH ratio with increasing oxygen concentrations used in resuscitation, although this trend was not significant.

Correlation between myocardial function and glutathione levels. Both cardiac index and SVI during reoxygenation correlated significantly with left ventricular myocardial total GSH levels. Figure 5-9 shows the positive correlation between left ventricular myocardial GSH and cardiac index ($r=0.61$, $p<0.005$) and SVI ($r=0.61$, $p<0.005$) at 30 minute of reoxygenation, with similar significant correlations seen throughout reoxygenation (data not

shown). Glutathione levels did not correlate with any other hemodynamic variables during reoxygenation, and did not correlate with any hemodynamic variables during hypoxia.

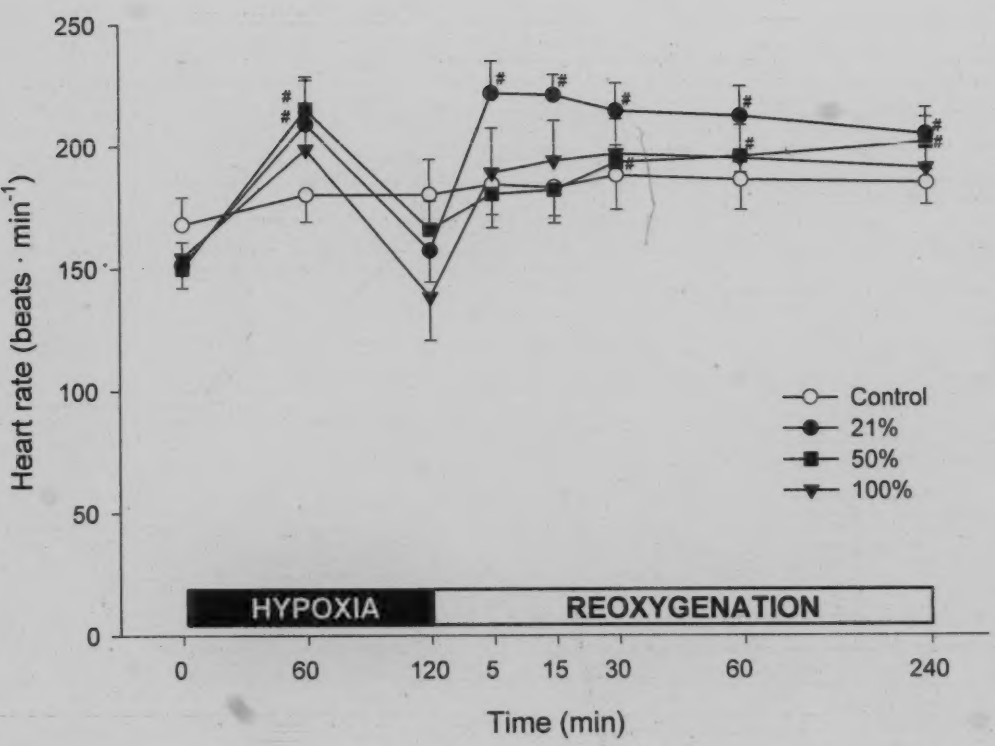
Histology. Varying degrees of intramural myocardial necrosis were observed in 6/8 hearts in the 21% group, 7/8 in the 50% group, and 6/8 in the 100% group, compared to 2/8 in controls. The degree of coagulation necrosis, cytoplasmic eosinophilia and nuclear pyknosis for each specimen is shown in figure 5-10. There were no significant differences in median histologic grade among individual groups, although significantly worse injury was observed when comparing all hypoxia-reoxygenated hearts to controls ($p<0.05$ for each histologic criteria). Figure 5-11 shows histologic samples from each group, demonstrating the pathologic criteria.

FIGURE 5-1. CARDIAC INDEX



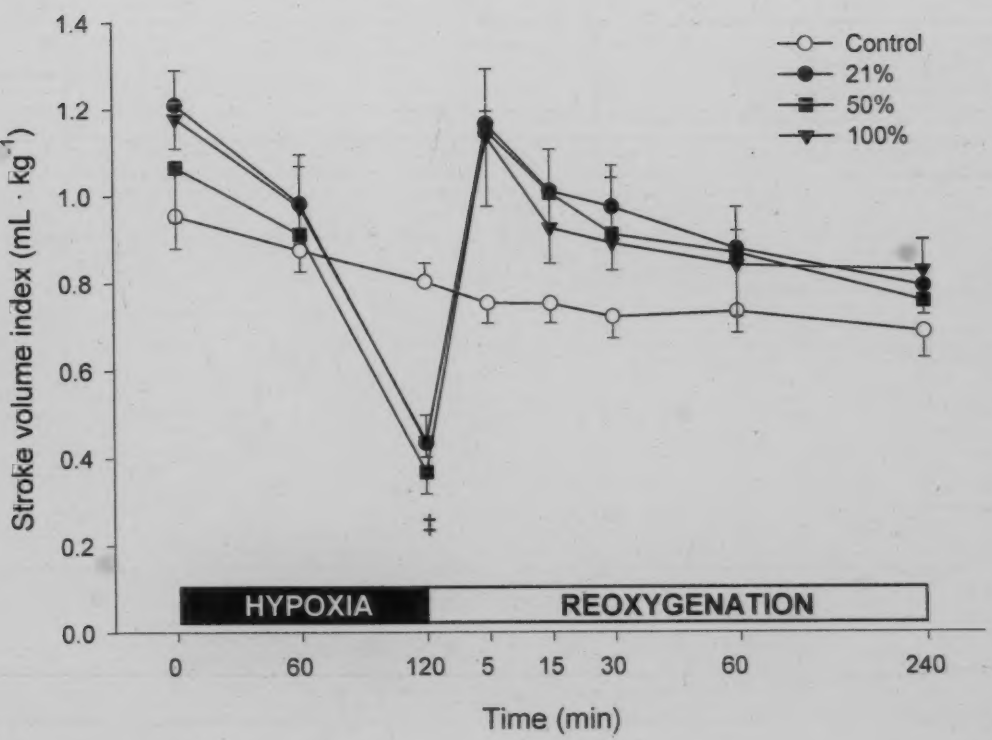
Cardiac index in piglets during 2 hours of hypoxia and 4 hours of reoxygenation with different oxygen concentrations. Control piglets underwent no hypoxia-reoxygenation. * $P<0.05$ vs. control. ‡ $P<0.001$ all hypoxic groups vs. control group.

FIGURE 5-2. HEART RATE



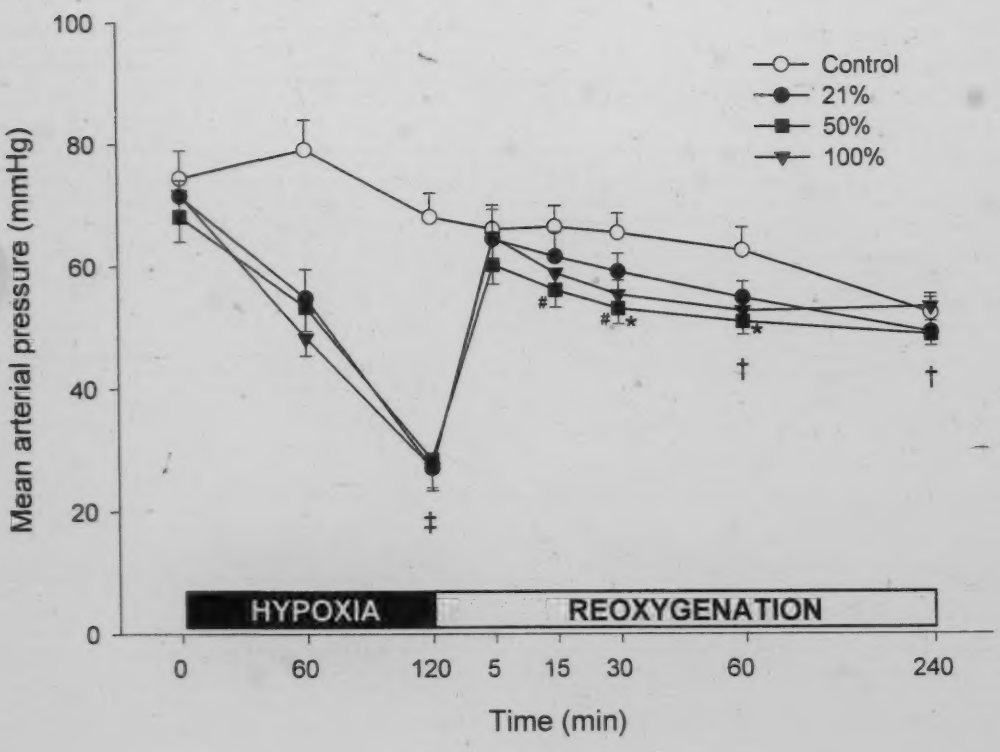
Heart rate in piglets during 2 hours of hypoxia and 4 hours of reoxygenation with different oxygen concentrations. Control piglets underwent no hypoxia-reoxygenation. # $P<0.05$ vs. baseline.

FIGURE 5-3. STROKE VOLUME INDEX



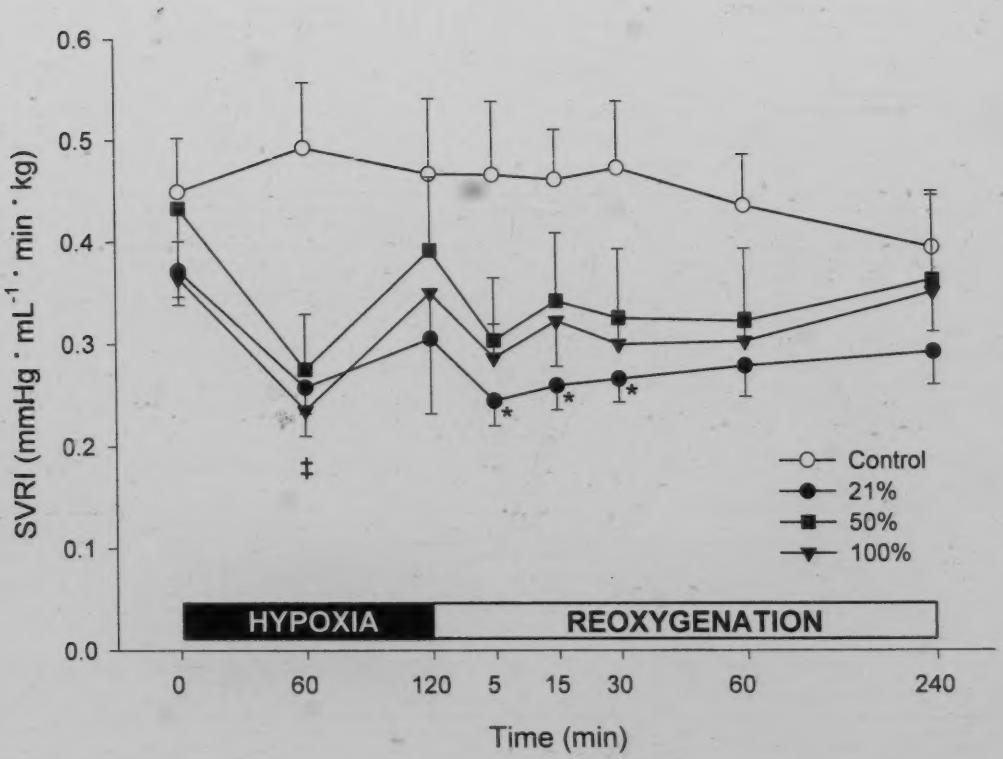
Stroke volume index in piglets during 2 hours of hypoxia and 4 hours of reoxygenation with different oxygen concentrations. Control piglets underwent no hypoxia-reoxygenation. ‡ $P<0.001$ all hypoxic groups vs. control group.

FIGURE 5-4. MEAN ARTERIAL PRESSURE



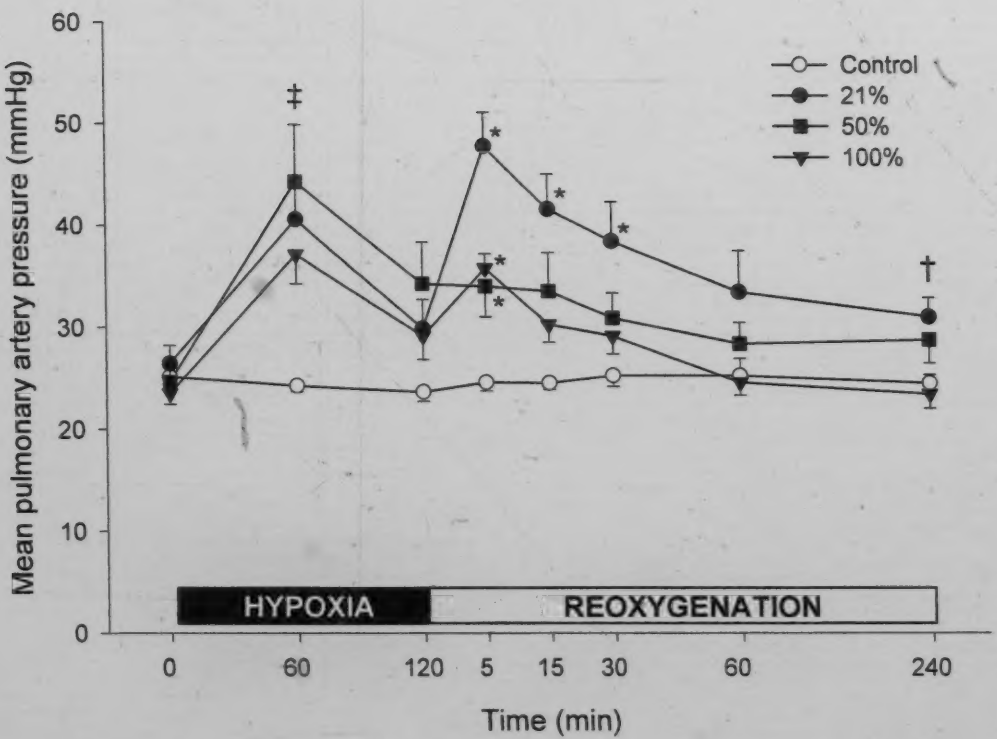
Mean arterial pressure during 2 hours of hypoxia and 4 hours of reoxygenation with different oxygen concentrations. Control piglets underwent no hypoxia-reoxygenation. ‡ P<0.001 all hypoxic groups vs. control group. * P<0.05 vs. control. # P<0.05 vs. baseline. † P<0.05 all groups vs. baseline.

FIGURE 5-5. SYSTEMIC VASCULAR RESISTANCE INDEX



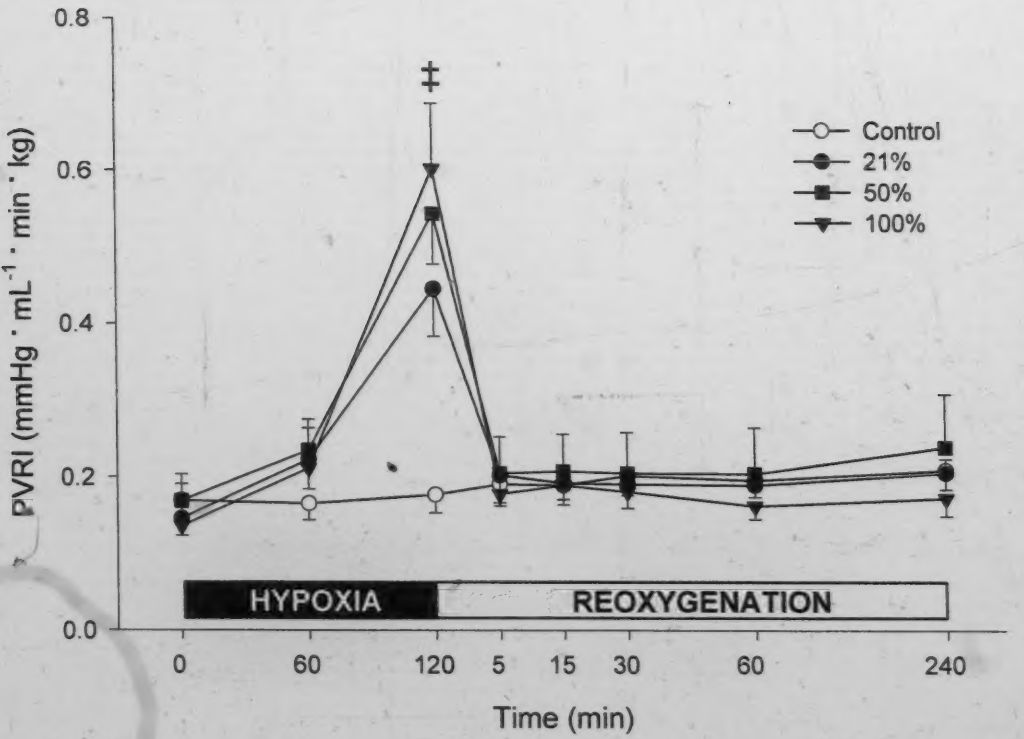
Systemic vascular resistance index (SVRI) during 2 hours of hypoxia and 4 hours of reoxygenation with different oxygen concentrations. Control piglets underwent no hypoxia-reoxygenation. ‡ $P<0.05$ all hypoxic groups vs. control group. * $P<0.05$ vs. control.

FIGURE 5-6. MEAN PULMONARY ARTERY PRESSURE



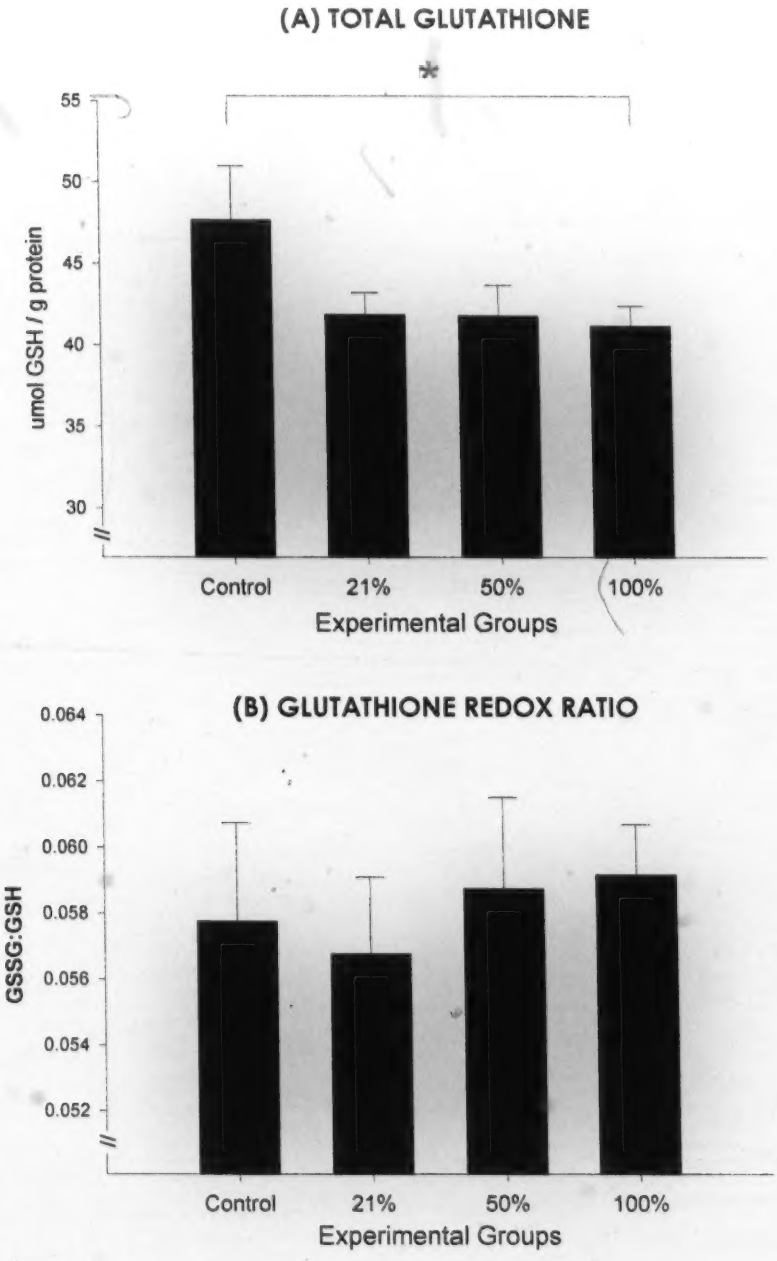
Mean pulmonary artery pressure during 2 hours of hypoxia and 4 hours of reoxygenation with different oxygen concentrations. Control piglets underwent no hypoxia-reoxygenation. ‡ $P<0.05$ all hypoxic groups vs. control group. * $P<0.05$ vs. control.

FIGURE 5-7. PULMONARY VASCULAR RESISTANCE INDEX



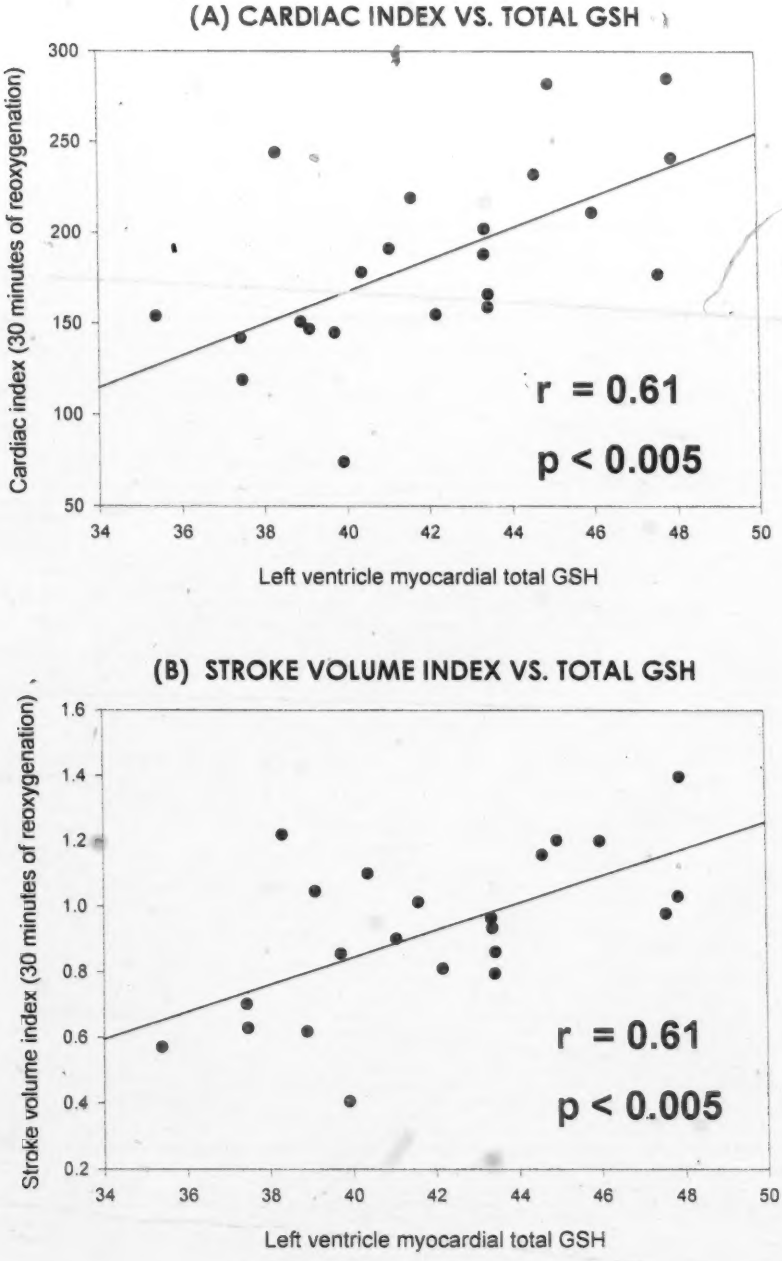
Pulmonary vascular resistance index (PVRI) during 2 hours of hypoxia and 4 hours of reoxygenation with different oxygen concentrations. Control piglets underwent no hypoxia-reoxygenation. ‡ $P<0.05$ all hypoxic groups vs. control.

FIGURE 5-8. LEFT VENTRICULAR MYOCARDIAL GLUTATHIONE



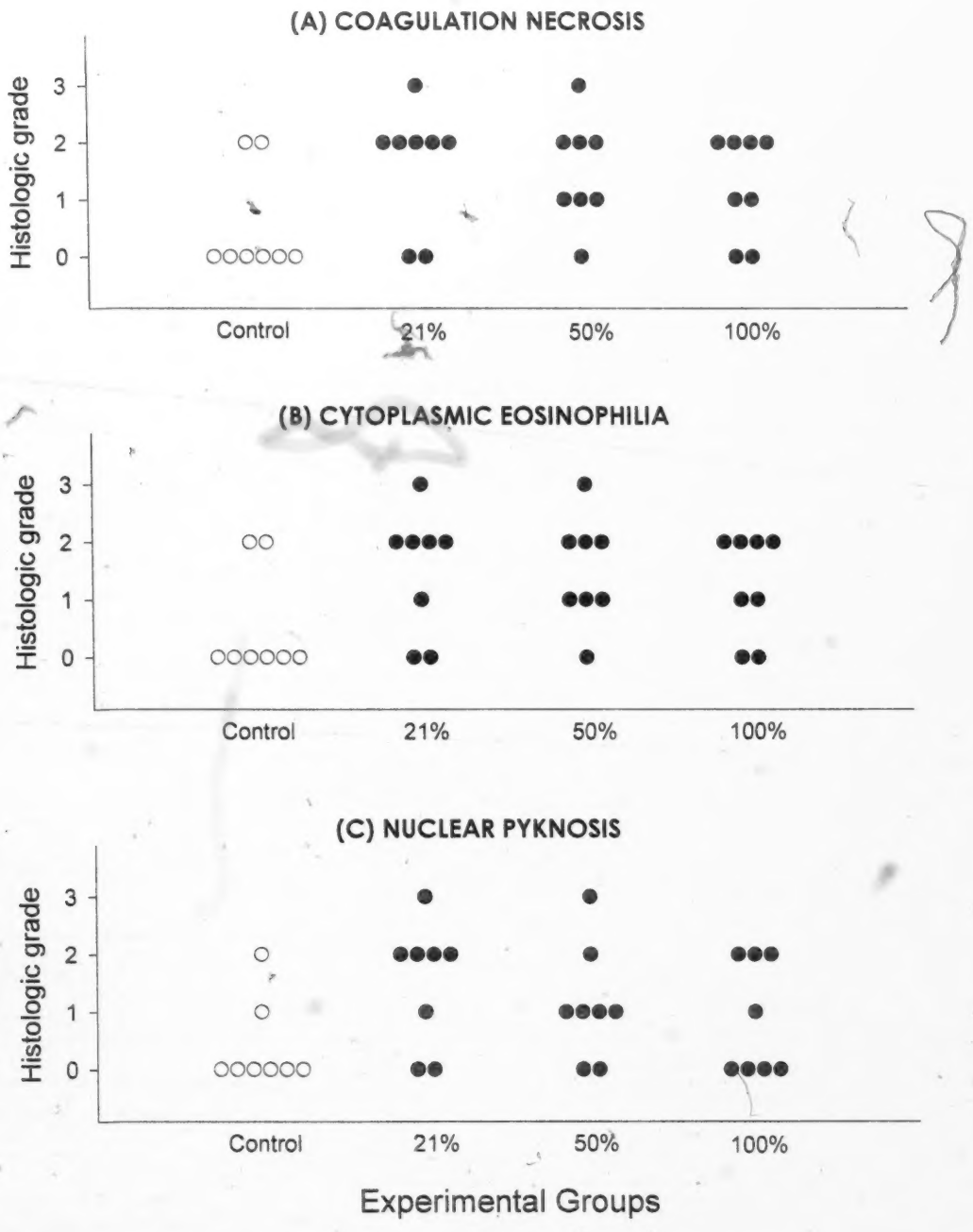
Left ventricular myocardial (A) total glutathione levels and (B) redox status (GSSG:GSH) in piglets following 2 hours of hypoxia and 4 hours of reoxygenation with different oxygen concentrations. Control piglets underwent no hypoxia-reoxygenation. * $P<0.05$.

FIGURE 5-9. MYOCARDIAL GLUTATHIONE CORRELATION WITH CARDIAC FUNCTION



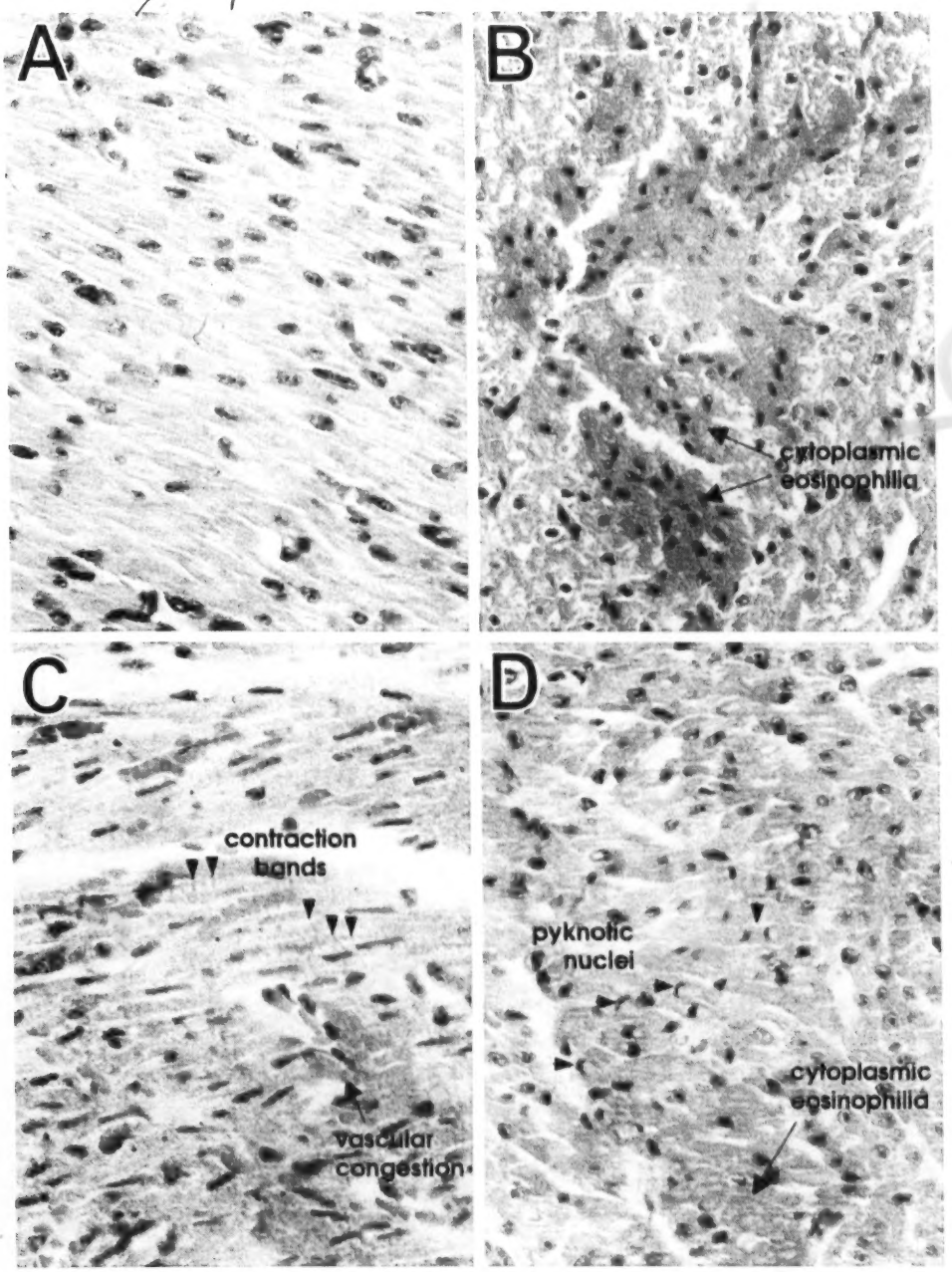
Pearson correlation between left ventricular myocardial total glutathione (GSH) and (A) cardiac index and (B) stroke volume index at 30 minutes of reoxygenation.

FIGURE 5-10. GRADE OF MYOCARDIAL TISSUE INJURY



Grade of histologic injury to left ventricular myocardium following hypoxia and reoxygenation with different oxygen concentrations. 0=normal, 1=1 area, 2=2 or more areas, 3=geographic distribution.

FIGURE 5-11. CARDIAC HISTOLOGY



Left ventricle myocardium (H&E stain), (A) control heart showing normal histology, and (B) 21% (C) 50% and (D) 100% demonstrating pathologic findings of cardiomyocyte necrosis used in histologic grading.

DISCUSSION

Our study has shown in a newborn piglet model of hypoxia-reoxygenation that the concentration of oxygen used during reoxygenation has significant effects on cardiac function, systemic and pulmonary vascular hemodynamics, and left ventricular myocardial GSH content. We have also shown that the level of myocardial GSH after 4 hours of reoxygenation correlates significantly with cardiac index and stroke volume measured during the entire reoxygenation period.

Histologic analysis showed that 19/24 hearts subjected to hypoxia-reoxygenation developed evidence of early myocardial necrosis, although we did not observe any significant difference between the reoxygenation groups. Interestingly, the necrotic areas were located intramurally, and not in the subendocardial area as would be expected in the setting of hypoxic-ischemic cardiac injury. Thus we speculate that this injury is indeed reoxygenation injury, and not hypoxic-ischemic injury, supported also by the findings of congestion in many specimens and contraction bands, as shown in figure 5-11(C), which are typical features of reoxygenation or reperfusion injury.[36]

Resuscitation of hypoxemic newborns with concentrations of oxygen other than 100% has been revisited over the past decade. In two prospective, randomized, controlled trials, severely asphyxiated neonates resuscitated with room air were shown to have an equivalent recovery of heart rate, oxygen saturation, and overall clinical outcome compared to neonates resuscitated with 100% oxygen.[30,31] In newborn animals, one study

demonstrated that reoxygenation of severely hypoxemic piglets with 21% oxygen resulted in a transient increase in cardiac output during early reoxygenation, which was not seen in piglets reoxygenated with 100% oxygen, while there were no other hemodynamic differences between the groups.[34] Another study demonstrated a transiently increased cardiac output in both 21% and 100%-reoxygenated piglets.[33] The current study has shown that cardiac index is increased in the 21% group during the first 30 minutes of reoxygenation. This corresponds to an increase in HR, a non-significant increase in stroke volume, and a decrease in SVRI during the first 30 minutes of reoxygenation in the 21% group. Although MAP immediately recovered with reoxygenation, it then gradually decreased in all groups, decreasing earlier in the 50% and 100% group than in controls or 21% group. Overall, systemic hemodynamic recovery and cardiac function are slightly improved in piglets resuscitated using 21% oxygen.

Our study has shown an increase in pulmonary artery pressure, but no change in PVRI during reoxygenation with 21% oxygen compared to 50% or 100% oxygen. Persistent pulmonary hypertension in the newborn is a potential complication of neonatal hypoxemia,[37] and must be considered when evaluating different methods of resuscitation which, as we have shown, result in significant differences in pulmonary hemodynamics. However persistent pulmonary hypertension is caused by an increase in pulmonary vascular tone,[37] whereas in the present study, elevated PAP was due to an increase in pulmonary artery flow with no change in PVRI.

The use of 100% oxygen in the resuscitation of hypoxemic newborns

has been shown to increase markers of oxidant stress in the blood, indicating an increase in OFR activity.[31] Kondo *et al.*[38] reported that reoxygenation of asphyxiated newborn piglets with 100% oxygen caused a significantly greater OFR production in the lungs than in piglets resuscitated with 21%. Kutzsche *et al.*[39] showed that 100% oxygen increases leukocyte OFR concentration in the cerebral circulation compared to 21% oxygen used in the resuscitation of hypoxemic piglets. Oxygen free radicals are highly reactive and cytotoxic molecules generated during reperfusion or reoxygenation following hypoxia-ischemia, and have been implicated in cardiac functional impairment and systemic vascular changes.[1-3,19] Garlick *et al.*[20] first demonstrated that oxygen is the essential element for the production of free radicals during cardiac reperfusion. Zweier[4] showed that superoxide-derived OFRs generated during cardiac reperfusion are responsible for an impairment in myocardial contractile function during early reperfusion. Bolli et al. [21] demonstrated OFR generation in an intact animal model of myocardial ischemia-reperfusion, and showed that after an initial burst of OFR formation, there is ongoing OFR generation for up to 3 hours of reperfusion, and that the OFRs contributed to the ongoing contractile dysfunction during the 3 hours of reperfusion. Furthermore, inhibiting OFR formation with OFR scavengers[6] or iron chelators[7,40] has been shown to improve myocardial function and decrease cardiomyocyte damage. Of particular interest is the generation of OFRs in the neonatal heart. Rowland *et al.*[13] reported that newborn rat hearts showed improved functional tolerance to ischemia-reperfusion, and attributed this to an increase in the endogenous OFR-scavenging enzyme

catalase, and a decrease in the OFR-generating enzyme xanthine oxidase in newborn compared to adult hearts. Kohman and Veit[12] reported an increase in OFR damage as measured by myocardial lipid peroxidation following ischemia-reperfusion in adult compared to neonatal rabbit hearts, and also reported improved functional recovery in neonatal hearts. Thus, there is substantial evidence that OFRs generated during reperfusion or reoxygenation contribute to myocardial dysfunction, in adult and neonatal animals.

In our study, we investigated OFR activity by measuring myocardial antioxidant status. The GSH antioxidant system is the primary defence mechanism of cardiomyocytes against OFRs,[27] whereby reduced GSH reacts directly with OFRs to detoxify them, with the concomitant formation of GSSG.[24] Myocardial GSH levels have been shown to decrease significantly during cardiac ischemia-reperfusion, associated with the release of GSH and GSSG in the coronary effluent.[25] Glutathione depletion in cultured endothelial cells exposed to OFRs has been shown to correlate with cell death.[41] Conversely, increasing GSH activity has been shown to improve cardiac function. Cheung *et al.*[28] reported that GSH treatment resulted in a concentration-dependent improvement in cardiac functional recovery following either ischemia-reperfusion or oxidant infusion. They further suggested that the protective action of GSH is mediated by a decrease in the potent oxidant peroxynitrite. Le *et al.*[27] reported that stimulation of the GSH redox cycle in cultured neonatal rat cardiomyocytes exposed to oxidant stress improved cardiomyocyte function, decreased cell death, and

decreased lipid peroxidation. In the present study we have shown a decrease in left ventricular myocardial total GSH levels after 2 hours of hypoxia and 4 hours of reoxygenation, and this decrease was only significant in the piglets resuscitated with 100% oxygen, compared to controls. There was also a trend toward an increase in the myocardial GSH redox ratio, a marker of oxidative stress, with increasing oxygen concentrations used in reoxygenation. The level of myocardial total GSH correlated significantly with myocardial function as measured by cardiac index and stroke volume during reoxygenation. To our knowledge, our study is the first to demonstrate the relationship between myocardial function and GSH levels in an intact neonatal animal model of hypoxia-reoxygenation, and furthermore the decreased levels of myocardial GSH and trend towards an increased redox ratio with the use of 100% oxygen indicates that reoxygenation with 100% oxygen does indeed cause increase oxidative stress in the neonatal piglet myocardium.

In conclusion, our study has demonstrated that hypoxemic newborn piglets resuscitated with 21% oxygen have some improvements in cardiac index during early reoxygenation, and that cardiac index during reoxygenation correlates significantly with left ventricular myocardial GSH content. These result may have some implications in neonatal resuscitation, in that 21% oxygen does not appear to have any detrimental effects in cardiac function or systemic and pulmonary hemodynamic function, and this supports the evidence that 21% oxygen may be used effectively instead of 100% oxygen in neonatal resuscitation.

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CHAPTER 6

Resuscitation with 100% Oxygen Causes Intestinal Glutathione Oxidation and Reoxygenation Injury in Asphyxiated Newborn Piglets

A version of this chapter is in review for publication in Annals of Surgery.

INTRODUCTION

Necrotizing enterocolitis (NEC) is a disease in newborns characterized by ischemic necrosis of the gastrointestinal tract.[1-3] The incidence of NEC has been increasing, and the mortality associated with it is reported from 10-66%.[4,5] The pathogenesis of NEC is still uncertain, however hypoxic-ischemic injury in the perinatal period has been suggested as a likely risk factor.[2,6] Human studies have reported intestinal injury occurring in 6-29% of severely asphyxiated neonates,[7,8] and newborn animal models of intestinal hypoxia-reoxygenation[9-11] and ischemia-reperfusion[12] have clearly demonstrated characteristic histopathologic lesions similar to those seen in NEC.

Recently it has been suggested that NEC may belong to the group of so-called "oxygen free radical diseases of the newborn".[13] Oxygen free radicals (OFRs) are highly cytotoxic molecules generated during the restoration of oxygenated blood flow following ischemia or hypoxia. Perinatal asphyxia is a hypoxic-ischemic event, and with subsequent resuscitation infants are at risk for OFR-related injury to the vital organs.[14] Oxygen free radicals have been well-established as the primary mediators of intestinal ischemia-reperfusion injury in adult animals,[15-21] however to our knowledge there are as yet no studies examining the role of OFRs in newborn animal models of intestinal hypoxia-reoxygenation or in NEC.

Due to the risk of OFR-related injury in asphyxiated newborns during resuscitation, recent studies have investigated the effects of different oxygen concentrations used in neonatal resuscitation. Both human[22,23] and animal[24]

studies have shown that the use of 21% oxygen is as effective or even better than 100% oxygen in the resuscitation of severely hypoxic newborns, in terms of recovery of cardiopulmonary function and organ perfusion. Furthermore infants resuscitated with 100% oxygen had elevated blood markers of oxidative stress including an increased oxidized-to-reduced glutathione ratio (GSSG:GSH),[23] which has been established as a marker for OFR-related cellular injury.[25,26]

We hypothesize that OFRs generated during reoxygenation play a role in mediating intestinal injury in the newborn following asphyxia, and that higher concentrations of oxygen during resuscitation may lead to an increase in OFR generation, and thus predispose to increasing incidence or degree of injury. Furthermore we hypothesize that resuscitation with lower oxygen concentrations is as effective as 100% oxygen in terms of restoration of intestinal blood flow and hemodynamic recovery.

The objectives of the present study were to compare the response of the neonatal piglet intestine to reoxygenation with 21%, 50% or 100% oxygen following severe asphyxia. This included characterization of intestinal blood flow response to hypoxemia and graded reoxygenation, evaluation of histopathologic changes, and measurement of oxidized and reduced glutathione (GSH) levels as a biochemical marker for OFR activity.

METHODS

Animals. Thirty-two newborn Yorkshire-Landrace piglets 1-3 days of age weighing 1.5-2.1 kg were obtained on the day of experimentation from a local farm. All experiments were conducted in accordance with the guidelines and approval of the Health Sciences Animal Policy and Welfare Committee of the University of Alberta.

Anesthesia. Anesthesia was induced with inhaled halothane at 5% which was then decreased to 2-3%. Once mechanical ventilation was commenced, halothane was discontinued and anesthesia was maintained with intravenous fentanyl 5-10 μg/kg/h, midazolam 0.1-0.2 mg/kg/h and pancuronium 0.05-0.1 mg/kg/h, with additional boluses as necessary, and acepromazine 0.25 mg/kg. Oxygen saturation was continuously monitored with a pulse oximeter (Nellcor, Hayward, CA), and heart rate and blood pressure were measured with a Hewlett Packard 78833B monitor (Hewlett Packard Co., Palo Alto, CA). Inspired oxygen concentration (F_iO_2) was measured by an Ohmeda 5100 oxygen monitor (Ohmeda Medical, Laurel, MD), and maintained at 0.21-0.24 for oxygen saturation between 90% and 100%. Maintenance fluids during experimentation consisted of 5% dextrose in water at 7.5 mL/kg/h and 0.9% NaCl at 2.5 mL/kg/h. Piglet temperature was maintained at 38.5-39.5°C using an overhead warmer and a heating pad.

Surgical procedure. A 5-French Argyle™ single lumen arterial catheter (Sherwood Medical Co., St. Louis, MO) was inserted into the distal aorta via the right femoral artery, and was attached to a pressure transducer and

monitor for continuous systemic arterial blood pressure measurements. A 5-French Argyle™ double lumen catheter was inserted to the level of the right atrium via the right femoral vein for the administration of medications and fluids. A tracheotomy and endotracheal intubation was performed, and pressure-controlled assisted ventilation was commenced (Sechrist infant ventilator model IV-100, Sechrist Industries Inc., Anaheim, CA) with pressures of 19/4 cm H_2O at a rate of 18-20 breaths/minute. The retroperitoneum was opened via a left flank incision and the superior mesenteric artery (SMA) was isolated with minimal dissection and encircled with a three-millimetre transit time ultrasound flow probe (3SB262, Transonic Systems Inc., Ithica, NY) attached to a Transonic T208 2-channel small animal blood flow meter for continuous measurement of blood flow. A left anterior thoracotomy in the third intercostal space was performed and a six-millimetre Transonic flow probe (6SB906) was placed around the main pulmonary artery to continuously measure cardiac output. All incisions were closed or covered to minimize evaporative heat loss.

Stabilization and Monitoring. Piglets recovered from the surgical procedure until baseline hemodynamic measures were stable (change less than ±10% over 20 minutes). Preliminary arterial blood gas analysis was performed at this time and the ventilator rate was adjusted as necessary to keep the P_aCO_2 30-45 mmHg. Mean systemic arterial blood pressure (MAP), heart rate, cardiac output, SMA flow, and oxygen saturation were continuously monitored and recorded throughout the experiment. Analogue outputs of the pressure amplifiers and flow monitors were digitized by a DT

2801-A analogue to digital converter board (Data translation, Ontario, Canada) in a Dell 425E personal computer. Software was custom written using the Asyst programming environment.

Experimental protocol. Piglets were block randomized into four groups (n=8 each). In three groups, hypoxemia was induced by ventilating the piglets with an F_iO_2 of 0.10 by increasing the concentration of inhaled nitrogen gas. The F_iO_2 was adjusted as necessary from 0.07 to 0.15 as tolerated by the piglet, to obtain a P_aO_2 of 20-40 mmHg for 2 hours. Following hypoxia, piglets were resuscitated with an F_iO_2 of 0.21 (21% group), 0.50 (50% group), or 1.0 (100% group) for 1 hour, followed by 0.21 for 3 hours. A control group of piglets was ventilated and oxygenated at an F_iO_2 of 0.21 for the 6 hours of study, with no period of hypoxia or reoxygenation. Hemodynamic and regional blood flow recordings were carried out at specified timepoints: baseline (0 minutes), 60 and 120 minutes of hypoxia, and 5, 10, 15, 30, 60, and 240 minutes of reoxygenation, and calculated as a mean over two minutes of recording. At the end of the experiment, piglets were euthanized with a bolus of 100 mg/kg pentobarbital.

Hemodynamic calculations. Hemodynamic variables were calculated as shown in Appendix 1 (page 177).

Blood and tissue collection. Arterial blood samples were drawn and immediately processed for gas and acid base analysis and hemoglobin measurements (Stat Profile, Nova Biomedical, Waltham, MA) at baseline, 30 and 120 minutes of hypoxia, and 30 and 240 minutes of reoxygenation. The measured hemoglobin values did not decrease significantly during the entire

experiment. Tissue was collected immediately after the piglets were euthanized. A sample of distal ileum, approximately 20 cm from the ileocecal valve, was collected and immediately snap-frozen in liquid nitrogen and stored at –80°C until biochemical analysis, while another sample was fixed in 10% formalin for histologic analysis.

Glutathione. Intestinal levels of total and oxidized GSH were measured using a GSH assay kit (catalog no. 703002, Cayman Chemical, Ann Arbor, MI). Briefly, frozen samples of ileal tissue were homogenized with 1 mL/100 mg of buffer containing 0.2 M 2-N-morpholino ethanesulphonic acid, 50 mM phosphate, and 1 mM EDTA, pH 6.0. Homogenates were centrifuged at 10 000 g for 15 minutes at 4°C, and the supernatant was collected and deproteinated with 10% metaphosphoric acid and 4 M triethanolamine, to avoid interference from sulfhydryl groups on proteins in the sample. A colorimetric microplate assay was performed by adding GSH reductase, $NADP^+$, and 5,5'-dithiobis-2-nitrobenzoic acid to the sample. The absorbance was measured after 25 minutes at 405 nm with a microplate reader (Spectra Max 190, Molecular Devices, Sunny Vale, CA), and the total GSH concentration was calculated from a standard curve. To measure oxidized glutathione (GSSG), deproteinated samples were incubated at room temperature for one hour with 1 M 2-vinylpyridine to completely derivatize the reduced GSH in the sample, and the colorimitric assay was carried out as above. Reduced GSH was calculated by subtracting GSSG from total GSH, and GSH redox status (GSSG:GSH) was obtained by calculating the ratio of oxidized-to-reduced GSH.

Histologic analysis and grading. Intestinal specimens preserved in formalin were prepared for routine histologic evaluation using hematoxylin and eosin staining, and for scanning electron microscopy. Sections were analyzed by two independent pathologists who were not aware of the study groups, and degree of histologic damage was graded by Park's classification of ischemic intestinal injury.[27]

Statistics. Results are expressed as mean ± standard error of the mean. The hemodynamic variables were analyzed by two-way repeated measures (RM) analysis of variance (ANOVA) followed by one-way RM ANOVA to determine differences over time compared to baseline values in each group, or one-way ANOVA to determine differences between groups at each timepoint, (SPSS 11.0.1, SPSS Inc., Chicago, IL, and Sigma Stat 2.0, Jandel Corp., San Rafael, CA). One-way ANOVA was used to determine differences in tissue GSH. ANOVA was carried out as above on ranks if tests of normality or equal variances failed. Post-hoc testing using the Tukey method for one-way ANOVA and RM ANOVA or Dunnett's method for ANOVA on ranks was used for pairwise comparisons. Pearson correlation analysis was used to assess the relationship between hemodynamic variables and GSH levels. Histologic injury was compared using Fisher's Exact test. Significance was defined as $p<0.05$.

RESULTS

Arterial blood gas analysis. Severe asphyxia was achieved in our piglets after exposure to hypoxia for 2 hours, as shown in table 6-1. P_aCO_2 was unchanged from baseline or control values. Upon reoxygenation, P_aO_2 was significantly higher in the 100% group compared to all other groups, and P_aO_2 in the 50 % group was greater than in the 21% group or controls. At the end of the study period, there were no significant differences in pH, P_aO_2, P_aCO_2, or bicarbonate among the different groups.

Hemodynamic variables. Piglets subjected to hypoxia developed shock and systemic hypotension, with cardiac output and MAP both decreasing to 41% ± 2% of control values after 2 hours of hypoxemia ($p<0.001$), as shown in table 6-2. Heart rate did not change significantly during the study period compared to controls. Upon reoxygenation, cardiac output rapidly recovered to baseline levels in the 50% and 100% groups, and in the 21% group, cardiac output significantly increased to 180% ± 14% of control values during the first five minutes of reoxygenation. Mean arterial pressure recovered immediately upon reoxygenation in all groups. Hemodynamic variables then remained similar to control values until the end of the study period in all experimental groups.

Superior mesenteric artery blood flow. Two hours of hypoxemia caused a marked decrease in SMA flow to 34% ± 3% of control values ($p<0.001$ vs. control group), as shown in figure 6-1, while SMA vascular resistance remained unchanged during hypoxia (figure 6-2). Upon

reoxygenation, SMA flow in the 21% group immediately increased to 177% ± 17% of the baseline value at 5 minutes of reoxygenation, and remained significantly elevated at 10 and 15 minutes of reoxygenation ($p<0.05$ vs. baseline), while SMA flow in the 50% and 100% groups transiently increased to 157% ± 21% and 145% ± 16% of baseline values respectively only at five minutes of reoxygenation ($p<0.05$). Superior mesenteric vascular resistance decreased in all groups during the first 30 minutes of reoxygenation, as shown in figure 6-2. There were no significant changes in SMA blood flow or vascular resistance in the control group throughout the study period.

Superior mesenteric oxygen delivery. Superior mesenteric oxygen delivery was severely impaired during hypoxemia, decreasing to 8.7% ± 2.6% of controls ($p<0.001$) as shown in figure 6-3. Upon reoxygenation, SMA oxygen delivery was significantly elevated above baseline values for the first 15 minutes of reoxygenation in the 100% group, and for the first 10 minutes in the 50% group ($p<0.05$). In the 21% group, oxygen delivery immediately recovered to baseline values. SMA oxygen delivery in control piglets did not show any significant changes throughout the study period.

Small intestinal glutathione. With increasing oxygen concentrations used in resuscitation, we observed a decreasing level of small intestinal total GSH (and reduced GSH, data not shown) and an increasing level of GSSG, although these differences were not statistically significant (figure 6-4). A similar increasing trend in GSSG:GSH was observed (data not shown). Small intestinal GSH levels in control piglets were 2.8 ± 0.3 μmol GSSG/g protein and 20.5 ± 1.7 μmol total GSH/g protein, which is comparable to levels previously

reported in 1- and 3-day-old piglets).[28]

Glutathione redox status and mesenteric oxygen delivery. Superior mesenteric oxygen delivery during early reoxygenation correlated positively with the GSSG:GSH ratio ($r = 0.59$, 0.58, and 0.58 at 5, 10 and 15 minutes of reoxygenation, respectively, $p<0.005$ for each correlation) as shown in figure 6-5, and also had a significant positive correlation with the level of intestinal GSSG ($p<0.05$, data not shown). Oxygen delivery during the hypoxic phase, and all other hemodynamic variables including SMA blood flow, cardiac output, or MAP had no significant correlation with GSH levels or redox status.

Histologic assessment. Gross evidence of intestinal necrosis with pneumatosis intestinalis was observed in segments of the terminal ileum in two piglets in the 100% group, while all other specimens were grossly normal ($p=0.056$, Fisher's Exact test, $\beta=0.46$). Microscopically, the two necrotic specimens demonstrated extensive subserosal and submucosal pneumatosis, as well as areas of complete villus denudation and destruction down to the crypt level as shown in figure 6-6. All specimens were graded by Park's criteria for intestinal tissue injury, and the results are shown in figure 6-7. Scanning electron micographs are shown in figure 6-8, demonstrating villus and microvillus changes following hypoxia and reoxygenation.

TABLE 6-1. ARTERIAL BLOOD GAS AND ACID BASE STATUS DURING HYPOXIA AND REOXYGENATION

		End Hypoxia	Reoxygenation	
Group	Baseline	120 min	30 min	240 min
pH				
Control	7.43 ± 0.02	7.44 ± 0.01	7.46 ± 0.03	7.40 ± 0.05
21%	7.48 ± 0.02	7.06 ± 0.04*	7.19 ± 0.03*	7.41 ± 0.02
50%	7.47 ± 0.03	7.06 ± 0.04*	7.15 ± 0.04*	7.38 ± 0.02
100%	7.46 ± 0.02	7.06 ± 0.04*	7.18 ± 0.04*	7.40 ± 0.01
P_aO_2 (mmHg)				
Control	83 ± 8	74 ± 5	86 ± 10	74 ± 5
21%	95 ± 11	33 ± 2*	69 ± 5	67 ± 5
50%	81 ± 6	32 ± 3*	189 ± 14†	70 ± 3
100%	90 ± 6	34 ± 2*	367 ± 15†	81 ± 7
P_aCO_2 (mmHg)				
Control	39 ± 2	39 ± 3	36 ± 2	42 ± 5
21%	34 ± 1	35 ± 1	35 ± 2	40 ± 2
50%	35 ± 3	37 ± 2	35 ± 2	37 ± 2
100%	37 ± 2	34 ± 3	32 ± 2	38 ± 2
Bicarbonate (mmol/L)				
Control	26 ± 2	27 ± 2	26 ± 2	25 ± 1
21%	26 ± 1	11 ± 1*	13 ± 1*	26 ± 1
50%	25 ± 1	11 ± 1*	12 ± 1*	23 ± 2
100%	26 ± 1	10 ± 1*	12 ± 1*	24 ± 1

All results are shown as mean ± SEM.

* P<0.001 vs. controls. † P<0.001 vs. all groups.

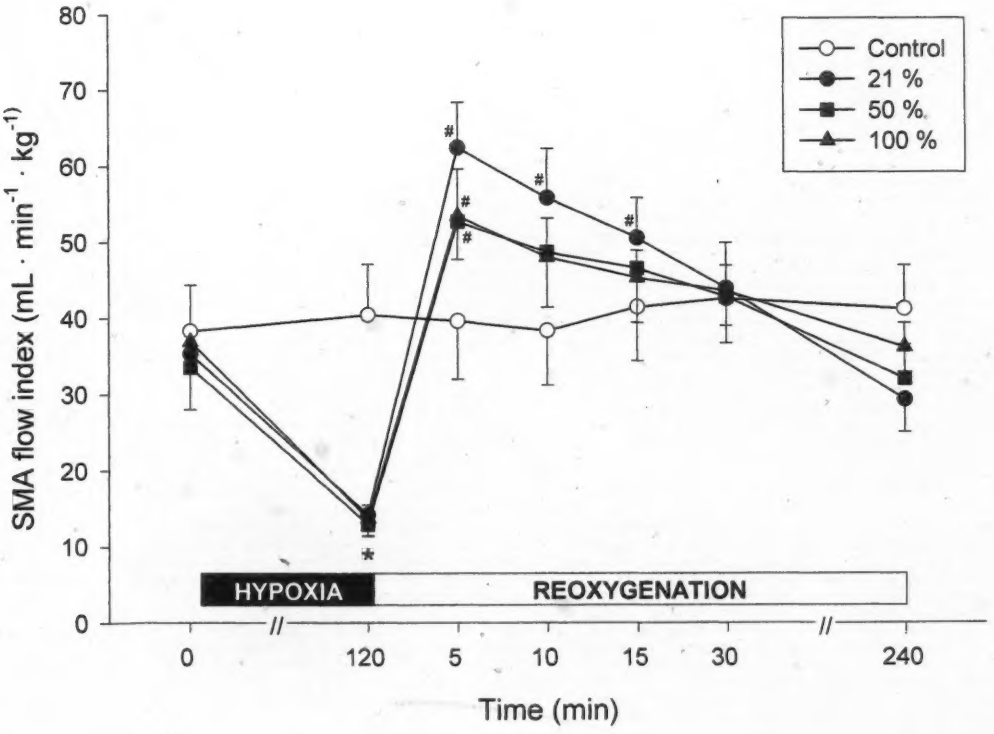
TABLE 6-2. HEMODYNAMIC VARIABLES DURING HYPOXIA AND REOXYGENATION

Group	Baseline	End Hypoxia	Reoxygenation		
		120 min	5 min	30 min	240 min
Heart rate (beats/min)					
Control	167 ± 11	180 ± 15	184 ± 18	188 ± 14	185 ± 9
21 %	151 ± 9	157 ± 13	221 ± 13	214 ± 11	204 ± 15
50 %	150 ± 8	166 ± 12	181 ± 8	194 ± 7	201 ± 14
100 %	154 ± 6	138 ± 18	189 ± 18	197 ± 14	191 ± 21
Mean arterial pressure (mmHg)					
Control	74 ± 5	68 ± 4	66 ± 4	66 ± 3	52 ± 2
21 %	72 ± 3	27 ± 4*	65 ± 5	59 ± 3	49 ± 2
50 %	68 ± 4	29 ± 1*	60 ± 3	53 ± 3†	49 ± 2
100 %	72 ± 4	27 ± 3*	65 ± 4	55 ± 2	53 ± 2
Cardiac output ($mL \cdot min^{-1} \cdot kg^{-1}$)					
Control	160 ± 15	145 ± 13	138 ± 12	136 ± 14	128 ± 14
21 %	180 ± 11	67 ± 5*	247 ± 20†	204 ± 17†	153 ± 12
50 %	160 ± 18	59 ± 7*	201 ± 28	172 ± 23	148 ± 21
100 %	181 ± 12	54 ± 7*	211 ± 19	176 ± 17	152 ± 22

All results are shown as mean ± SEM.

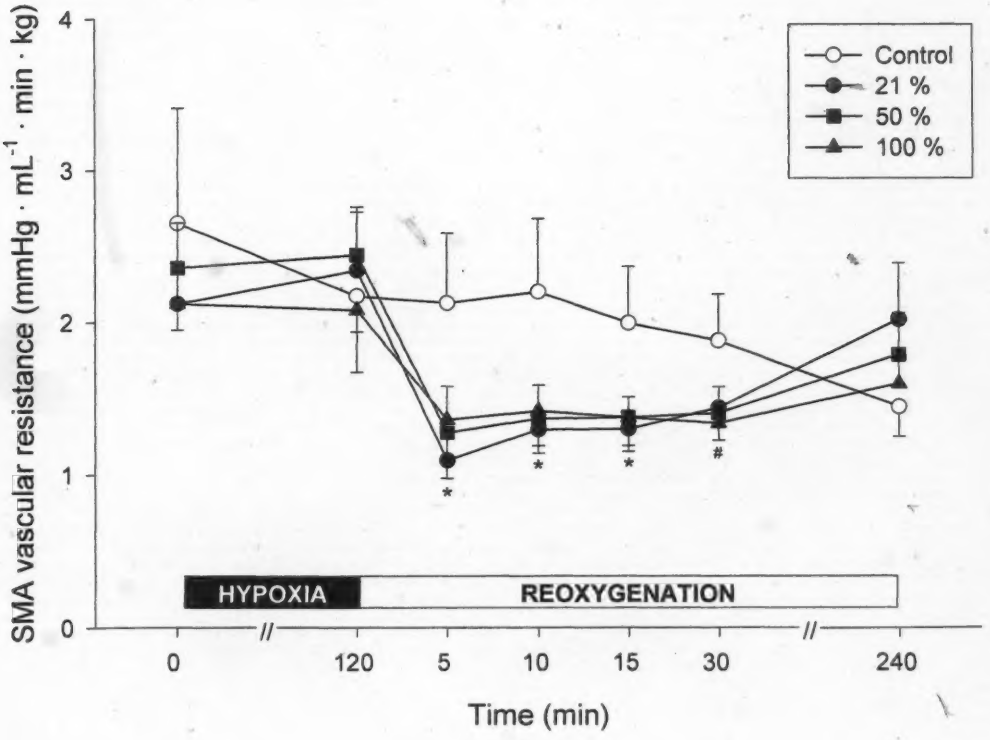
* $P < 0.001$ and † $P < 0.05$ vs. control group, one-way ANOVA.

FIGURE 6-1. SUPERIOR MESENTERIC ARTERY FLOW



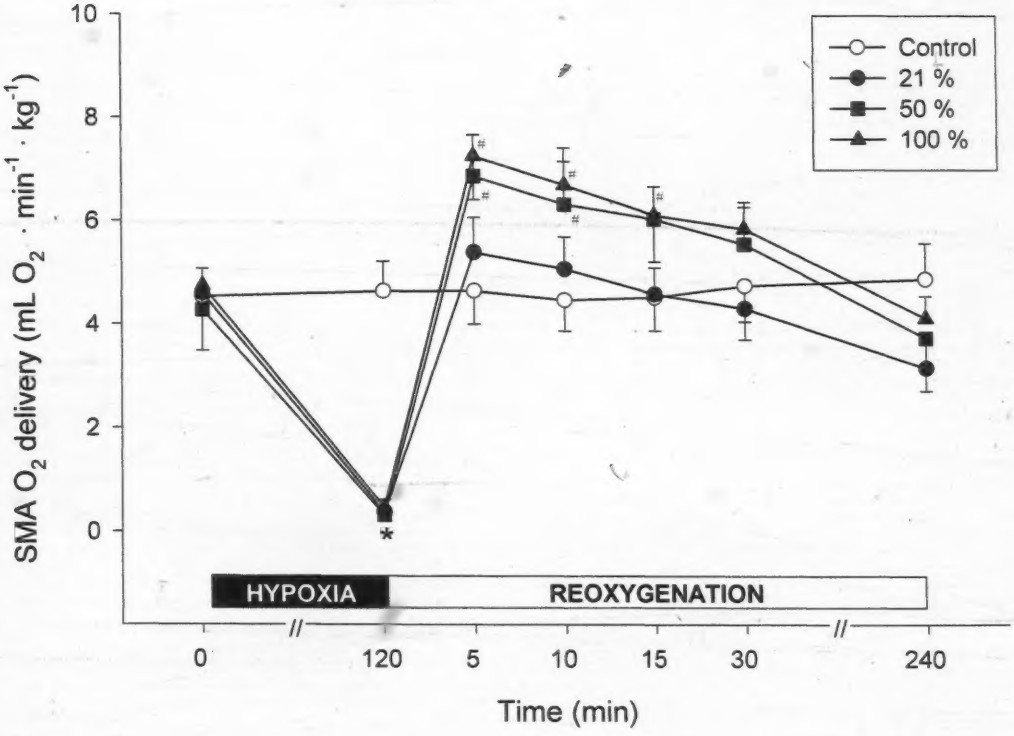
Superior mesenteric artery (SMA) flow index during hypoxia and reoxygenation with different oxygen concentrations. Control piglets (sham operated) underwent no hypoxia or reoxygenation. * $P<0.001$ each hypoxic group vs. control group, one-way ANOVA. # $P<0.05$ vs. baseline, one-way RM ANOVA.

FIGURE 6-2. SUPERIOR MESENTERIC VASCULAR RESISTANCE



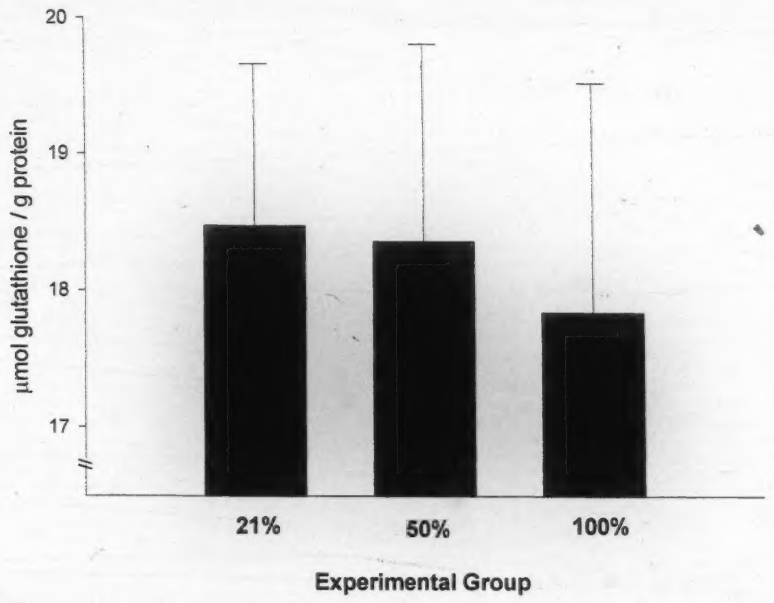
Superior mesenteric artery (SMA) vascular resistance index during hypoxia and reoxygenation with different oxygen concentrations. Control piglets (sham operated) underwent no hypoxia or reoxygenation. * $P<0.05$ each hypoxic group vs. baseline, one-way RM ANOVA. # $P<0.05$ in the 50% and 100% groups vs. baseline, one-way RM ANOVA.

FIGURE 6-3. SUPERIOR MESENTERIC OXYGEN DELIVERY

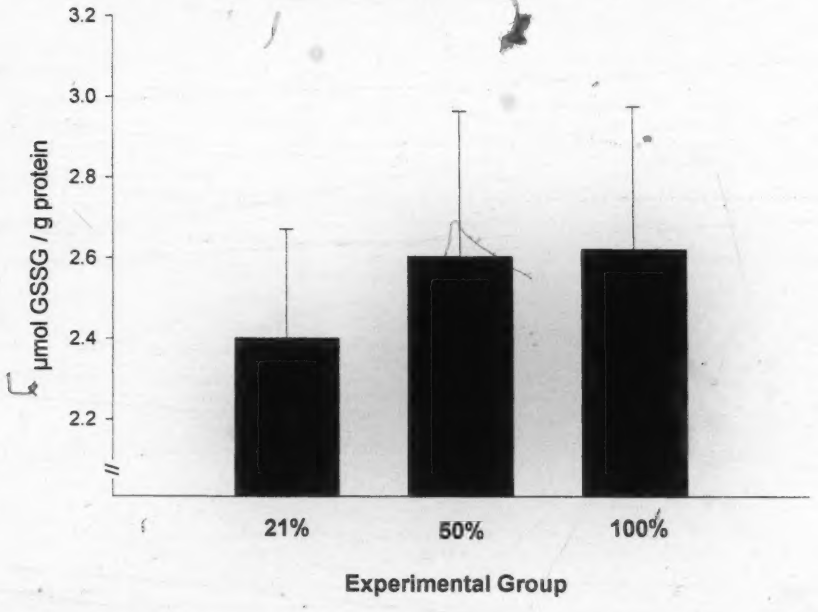


Superior mesenteric artery (SMA) oxygen delivery index during hypoxia and reoxygenation with different oxygen concentrations. Control piglets (sham operated) underwent no hypoxia or reoxygenation. * $P<0.001$ each hypoxic group vs. control, one-way ANOVA. # $P<0.05$ vs. baseline, one-way RM ANOVA.

FIGURE 6-4(A) INTESTINAL TOTAL GLUTATHIONE LEVELS

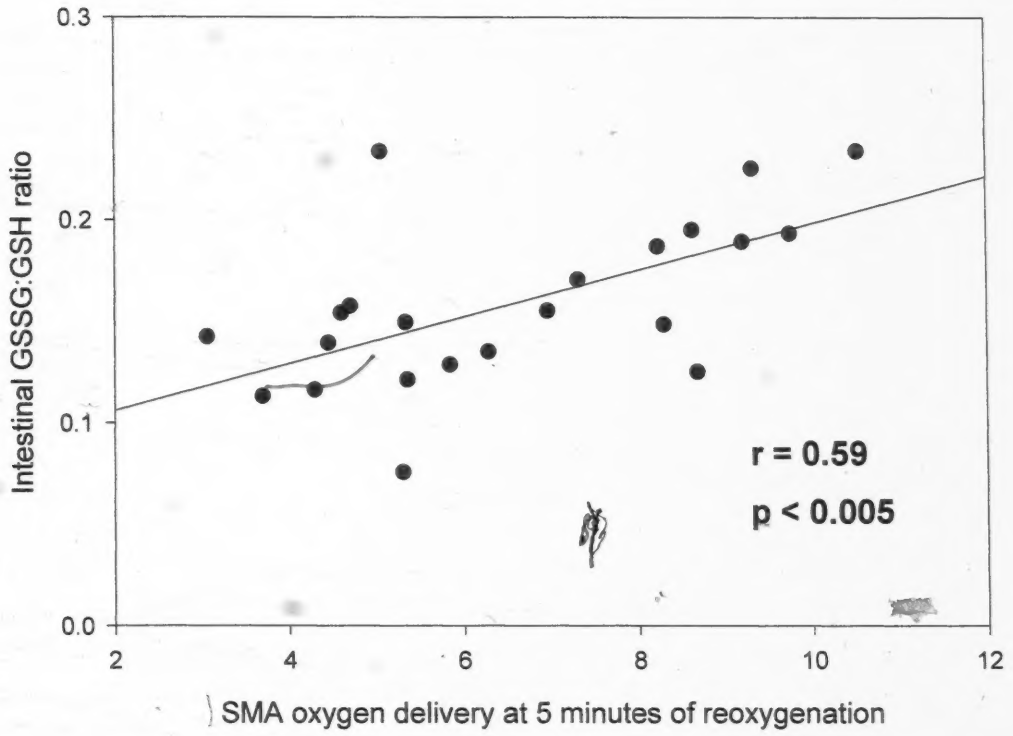


(B) INTESTINAL OXIDIZED GLUTATHIONE LEVELS



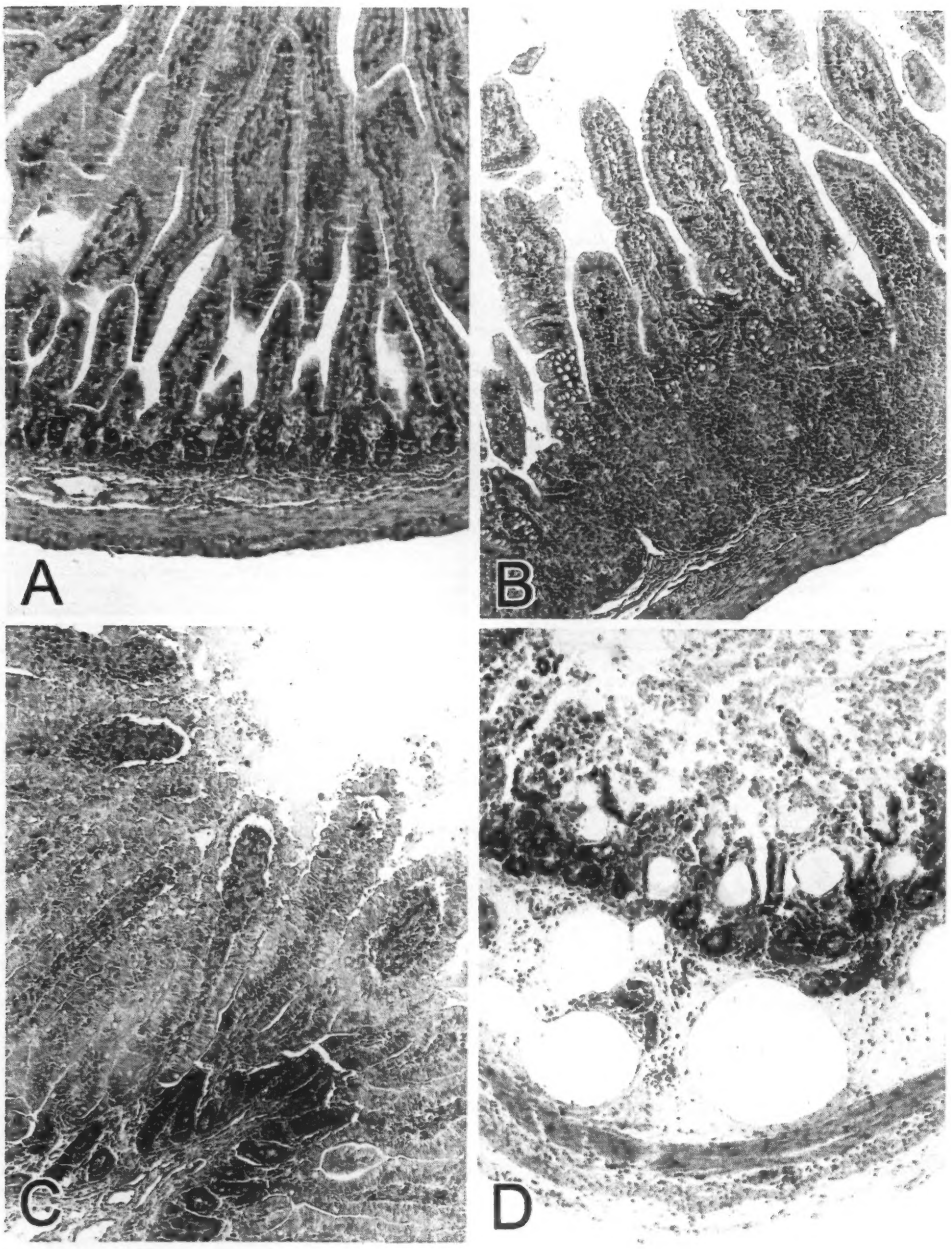
(A) Total glutathione (GSH) and (B) oxidized glutathione (GSSG) in piglet small intestine after 120 minutes of hypoxemia followed by 240 minutes of reoxygenation with different oxygen concentrations.

FIGURE 6-5. MESENTERIC OXYGEN DELIVERY AND INTESTINAL GLUTATHIONE REDOX RATIO CORRELATION



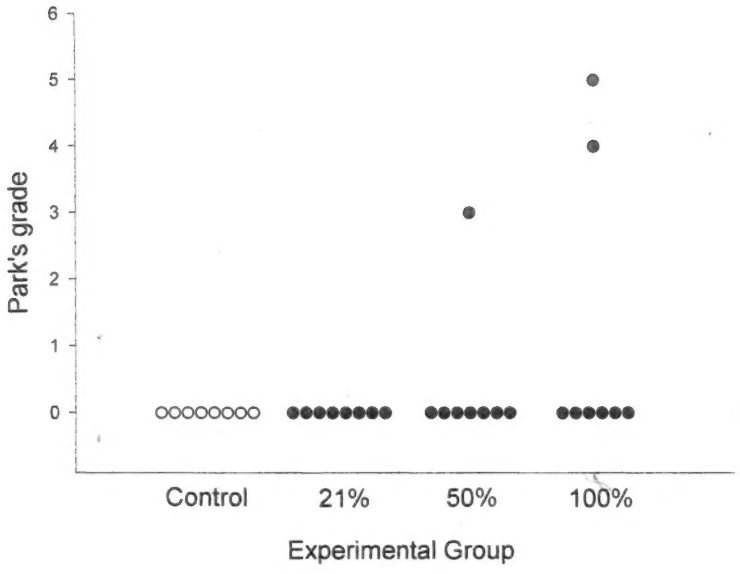
Correlation between superior mesenteric arterial (SMA) oxygen delivery at 5 minutes of reoxygenation and glutathione redox status (GSSG:GSH ratio) in piglet small intestine. Regression line and correlation coefficient (r) demonstrate a significant linear relationship between intestinal oxygen delivery and GSSG:GSH ratio.

FIGURE 6-6. SMALL INTESTINE HISTOLOGY



Histologic section (H&E stain) of terminal ileum from (A) sham-operated control piglet, and piglets exposed to 2 hours of hypoxia followed by 4 hours of reoxygenation with (B) 21%, (C), 50% and (D) 100% oxygen. Examples shown represent the worst histologic damage seen in each group.

FIGURE 6-7. GRADE OF INTESTINAL INJURY

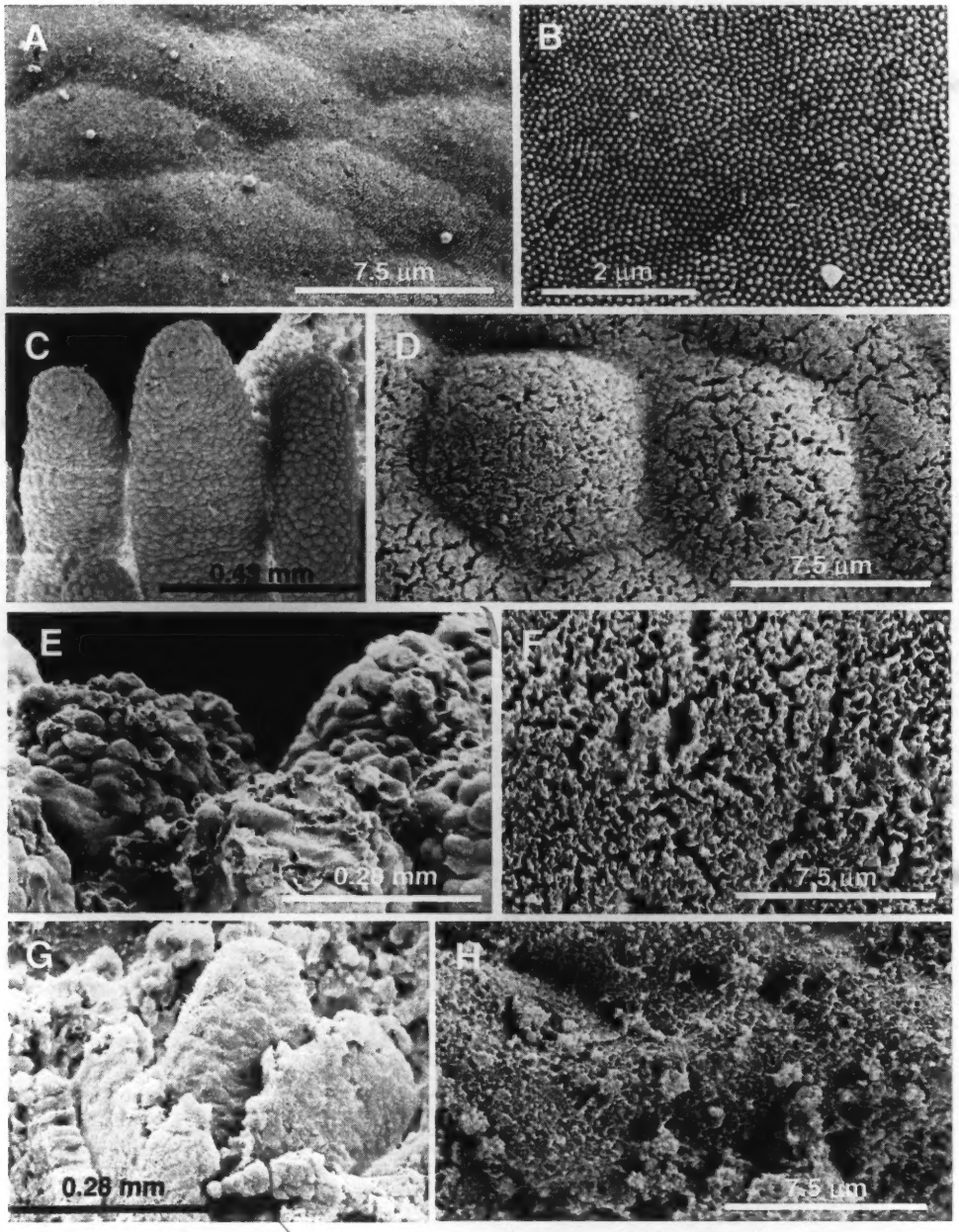


Degree of intestinal tissue injury following 2 hours of hypoxia and 4 hours of reoxygenation with different oxygen concentrations. Control piglets (sham operated) underwent no hypoxia or reoxygenation.

Park's microscopic criteria for the grading of intestinal injury:[27]

0 Normal mucosa
1 Subepithelial space at villus tip
2 Extended subepithelial space
3 Epithelial lifting along villus sides
4 Denuded villi
5 Loss of villus tissue
6 Crypt layer infarction
7 Transmucosal infarction
8 Transmural infarction.

FIGURE 6-8. SCANNING ELECTRON MICROGRAPH OF INTESTINAL MUCOSA



Scanning electron micrographs of intestinal villi and microvilli. (A) shows the luminal surface of enterocytes on a normal villus in a control piglet, and (B) shows multiple microvilli in a normal, uniform pattern on the luminal surface in a control piglet. (C) demonstrates intact villi and (D) shows enterocytes with subtle changes in microvillus uniformity in a representative piglet from the 21% group. (E) shows damaged villi with some lifting and separation of enterocytes, and (F) shows microvilli with some slight disruption in uniformity in a piglet from the 50% group. (G) shows severely damaged villi with sloughing of enterocytes, and (H) villus surface with debris covering the microvilli in a piglet from the 100% group.

DISCUSSION

Our study demonstrates that severe hypoxemia in newborn piglets results in significant impairment of intestinal perfusion and oxygenation, and that high oxygen concentrations used in the resuscitation of these piglets may predispose to intestinal lesions similar to those seen in NEC. Furthermore evidence of intestinal oxidative stress as a result of high mesenteric oxygen delivery suggest that OFRs may play a role in the pathogenesis of neonatal intestinal hypoxia-reoxygenation injury.

Necrotizing enterocolitis is the most common gastrointestinal emergency in neonates with an overall incidence of 1.1 per 1000 live births.[2,4] In 1969, Lloyd[29] first reported the high prevalence of asphyxia in neonates with apparently "spontaneous" gastrointestinal perforations, however the association between perinatal asphyxia and NEC remains controversial.[4,5,30-34] A recent prospective study of 72 term newborns with perinatal asphyxia found that 29% of infants had gastrointestinal manifestations, meeting Bell's[35] criteria for suspected NEC, although none had clinical and radiologic signs of definite NEC.[8] The pathogenesis of NEC is likely multifactorial, and intestinal hypoxia-ischemia in the neonatal period has been suggested to be an important factor.[3,6,33] A review of pathologic findings in 84 infants with NEC demonstrated ischemic necrosis in 90% of intestinal specimens from autopsy or surgery, with mucosal ulcerations, intestinal perforations, and pneumatosis intestinalis.[3] Overall the histologic damage seen in NEC is almost identical to other ischemic intestinal insults, although pneumatosis intestinalis is much more commonly seen in NEC.

While an ideal animal model of NEC remains to be established, newborn animal models of hypoxia-reoxygenation have helped to understand the pathogenesis of intestinal hypoxic-ischemic disease in the neonate. Several researchers have examined the relationship between intestinal hypoxia, blood flow, and the development of hypoxic-ischemic lesions similar to those seen in NEC.[9,11,36,37] Alward et al[9] and Karna et al[11] both demonstrated a significant decrease in blood flow to the gastrointestinal tract after 90 minutes of severe asphyxia in newborn piglets. Histologically they showed evidence of patchy mucosal necrosis[9] and findings similar to NEC including pneumatosis intestinalis in all piglets.[11]

The results of our study are consistent with previous reports,[9,11,36,37] demonstrating a significant decline in SMA blood flow (34% of control values) during severe hypoxemia. We have also demonstrated that during reoxygenation, there is a significant increase in SMA blood flow above baseline values, and furthermore that with lower oxygen concentrations used in resuscitation, the magnitude of increased SMA blood flow is greater, up to 177% of baseline blood flow with 21% resuscitation. Previous reports of intestinal blood flow during reoxygenation are contradictory, showing either a hyperemic response during reoxygenation following hypoxia,[24,36] a return to pre-hypoxemic baseline blood flow,[38] or a decrease in mesenteric flow during the reoxygenation period.[39] These studies however did not measure blood flow continuously and thus may have missed the peak flow, and none of the studies compared the effects of different oxygen concentrations. Our study has further demonstrated that increased intestinal flow is a result of both

decreased mesenteric vascular resistance and increased cardiac output during resuscitation. Restoration of blood flow is essential in the recovery from hypoxia-ischemia, and increased blood flow upon reoxygenation may be beneficial, as demonstrated in one previous study demonstrating that areas of the gastrointestinal tract with better perfusion following severe hypoxemia had less histologic injury.[36]

In our study, we have demonstrated in newborn piglets subjected to hypoxia and reoxygenation the development of intestinal lesions which are similar to those seen in NEC. Unique to our study is the demonstration that grossly necrotic lesions and pneumatosis intestinalis occur more frequently in piglets resuscitated with 100% oxygen, although this does not reach statistical significance ($p=0.056$). We have shown that piglets resuscitated with 100% oxygen have a significantly higher mesenteric oxygen delivery during the first 15 minutes of reoxygenation, and interestingly that increased oxygen delivery significantly correlates with intestinal oxidative stress as measured by the GSH redox ratio. In light of these findings, we suggest that OFRs play a role in the pathogenesis of intestinal injury in our newborn piglet model, and perhaps in NEC. It is well-established that OFRs play an important role in mediating intestinal injury following ischemia-reperfusion in adult animal models,[14-17,20,40-42] and it has been hypothesized that OFRs are responsible for a number of specific "oxygen radical diseases of the newborn" following perinatal asphyxia, such as bronchopulmonary dysplasia, retinopathy of prematurity, periventricular leukomalacia, patent ductus arteriosus, and NEC.[13,43] However to our knowledge there have been no previous studies

demonstrating OFR involvement in the pathogenesis of NEC or newborn animal models of intestinal hypoxia-reoxygenation.

In the present study, we used the intestinal GSH redox ratio as a marker for OFR activity. Glutathione is one of the most important endogenous cellular antioxidants during periods of oxidative stress, in which GSH is reversibly oxidized to GSSG in the detoxification of OFRs.[25,44,45] Previous studies have established the GSH redox status as a marker for increased oxidative stress.[23,25,26] In a prospective, randomized trial comparing the resuscitation of severely asphyxiated newborns with 100% versus 21% oxygen, Vento et al[23] demonstrated an increase in the GSSG:GSH ratio in blood samples at birth and 72 hours after birth in asphyxiated newborns compared to controls. Furthermore the GSSG:GSH ratio was higher in infants resuscitated with 100% oxygen compared to 21% oxygen, and clinically, newborns resuscitated with 21% oxygen recovered from the asphyxial event earlier than infants resuscitated with 100% oxygen. Our present study demonstrated that the intestinal GSSG:GSH ratio significantly correlated with SMA oxygen delivery during the first 15 minutes of reoxygenation, indicating that even a short period of exposure to high oxygen concentrations following hypoxemia can lead to increased tissue oxidative stress. The effect could be detrimental, as the GSH redox status has been shown to correlate with mitochondrial DNA damage[26] and lipid peroxidation.[45] The absolute levels of intestinal GSH and GSSG however did not differ significantly among the experimental groups, although there was a slight trend towards an increase in GSSG and a decrease in total GSH with increasing oxygen concentrations. This may be

due to the fact that intestinal tissue was collected four hours after reoxygenation, when oxygen delivery no longer differed between the groups and all piglets had been switched to 21% oxygen for 3 hours, thus allowing time for cellular antioxidant capacity to be replenished by the reduction of GSSG back to GSH.[44]

In conclusion, our study is the first to provide evidence of increased oxidative stress in the small intestine of asphyxiated newborn piglets resuscitated with 100% oxygen. Furthermore we have shown that resuscitation with 100% oxygen may predispose to the development of small intestinal tissue injury similar to that seen in NEC, while resuscitation of severely hypoxic piglets with lower oxygen concentrations have no detrimental systemic or mesenteric hemodynamic effects. The pathogenesis of NEC is not yet fully understood, but we propose that OFRs likely play an important role in mediating tissue injury, similar to that seen in adult intestinal ischemic disease. Our own study supports the evidence in humans and animals that during newborn resuscitation, high oxygen concentrations should be used with caution, as potentially detrimental effects may occur in the setting of increased oxygen delivery to the vital organs.

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CHAPTER 7

Glutathione Oxidation, Myeloperoxidase Activity, and Lipid Peroxidation in Newborn Piglet Lung Following Asphyxia and Reoxygenation with 21%, 50% or 100% Oxygen

A version of this chapter has been submitted for publication to Pediatric Research.

INTRODUCTION

Oxygen free radicals (OFRs) have been implicated as important mediators of oxygen toxicity in the lung.[1,2] Exposure to high oxygen concentrations for brief periods leads to dyspnea, tachypnea, decreased vital capacity and atelectasis, while prolonged oxygen exposure can result in damage to alveolar cells and capillary endothelium, neutrophil infiltration, and alveolar and interstitial edema.[1] Animal experiments have shown that tracheal instillation of free-radical generating enzymes to normal rat[3] and guinea pig[4] lungs caused severe acute lung injury and reduced lung compliance, which was prevented by the administration of the OFR scavenger superoxide dismutase. Furthermore, intravenous administration of the OFR precursor hypoxanthine during exposure to 100% oxygen was shown to cause more severe oxygen toxicity in rats compared to either hypoxanthine or 100% oxygen alone.[5] Free radical generation has been demonstrated *in vivo* following asphyxia and resuscitation in newborn piglets, whereby resuscitation with 100% oxygen resulted in significantly greater free radical generation from the lung surface compared to resuscitation with 21% oxygen.[6]

Newborns may be at a higher risk of oxygen toxicity compared to adults, due to an immaturity in antioxidant defences.[7] Since bronchopulmonary dysplasia was first reported in 1967,[8] it has been recognized that prolonged exposure to high concentrations of oxygen is an important risk factors for the progression to chronic lung disease (CLD).[9-11]

Recent studies have shown that newborns who go on to develop CLD have higher plasma levels of oxidation products within 24-48 hours after birth, compared to infants with respiratory distress syndrome who do not progress to CLD,[12,13] suggesting that OFRs are likely involved in the pathogenesis of lung injury. Neutrophil infiltration has also been implicated as an important contributor to the development of lung injury, and has been shown to correlate with degree of lipid peroxidation and protein oxidation in the lungs of newborns with respiratory distress.[14]

Resuscitation of newborns with respiratory distress traditionally involves the administration of 100% oxygen,[15] thus immediately exposing newborns to the potentially detrimental effects of OFRs. Recent prospective trials however have shown that resuscitation of severely asphyxiated newborns with room air is as effective as 100% oxygen,[16,17] and furthermore, that infants resuscitated with 100% oxygen had higher levels of oxidative stress in their blood.[18] We hypothesize that resuscitation with higher oxygen concentrations results in more tissue oxidative stress, neutrophil infiltration, and OFR activity, and therefore more severe injury in the lung, in the acute setting.

In the present study, our objective was to determine if the concentration of inspired oxygen used during resuscitation of asphyxiated newborn piglets had an influence on glutathione oxidation, myeloperoxidase activity, and lipid peroxidation in the lung.

METHODS

Animals. All experiments were conducted in accordance with the guidelines and approval of the Health Sciences Animal Policy and Welfare Committee of the University of Alberta. Thirty-two newborn Yorkshire-Landrace piglets 1-3 days of age weighing 1.5-2.1 kg were obtained on the day of experimentation from a local farm.

Surgical procedure. Anesthesia was induced with inhaled halothane at 5% which was then decreased to 2-3%. A 5-French Argyle™ single lumen arterial catheter (Sherwood Medical Co., St. Louis, MO) was inserted into the distal aorta via the right femoral artery, and was attached to a pressure transducer and monitor (Hewlett Packard 78833B monitor, Hewlett Packard Co., Palo Alto, CA) for systemic arterial blood pressure monitoring. A 5-French Argyle™ double lumen catheter was inserted to the level of the right atrium via the right femoral vein for the administration of medications and fluids. A tracheotomy and endotracheal intubation was performed, and pressure-controlled assisted ventilation was commenced (Sechrist infant ventilator model IV-100, Sechrist Industries Inc., Anaheim, CA) with pressures of 19/4 cm H_2O at a rate of 18-20 breaths/minute. Oxygen saturation was measured with a pulse oximeter (Nellcor, Hayward, CA), and inspired oxygen concentration (F_iO_2) measured by an Ohmeda 5100 oxygen monitor (Ohmeda Medical, Laurel, MD), was maintained at 0.21-0.24 for oxygen saturation between 90% and 100%. Halothane was then discontinued and anesthesia was maintained with intravenous fentanyl 5-10 μg/kg/h, midazolam 0.1-0.2 mg/kg/h and pancuronium 0.05-0.1 mg/kg/h, with additional boluses as

necessary, and acepromazine 0.25 mg/kg. A left anterior thoracotomy in the third intercostals space was performed and a six-millimetre transit time ultrasound flow probe (6SB906, Transonic Systems Inc., Ithica, NY) was placed around the main pulmonary artery and attached to a Transonic T206 2-channel small animal blood flow meter to continuously monitor cardiac output. Maintenance fluids during experimentation consisted of 5% dextrose in water at 7.5 mL/kg/h and 0.9% NaCl at 2.5 mL/kg/h. Piglet temperature was maintained at 38.5-39.5°C using an overhead radiant warmer and a heating pad.

Stabilization and Monitoring. Piglets recovered from the surgical procedure until baseline hemodynamic measures were stable (change less than ±10% over 20 minutes). Preliminary arterial blood gas analysis was performed at this time and the ventilator rate was adjusted as necessary to keep the P_aCO_2 30-45 mmHg. Mean arterial blood pressure (MAP), cardiac output, and oxygen saturation were continuously monitored and recorded throughout the experiment. Analogue outputs of the pressure amplifiers and flow monitors were digitized by a DT 2801-A analogue to digital converter board (Data translation, Ontario, Canada) in a Dell 425E personal computer. Software was custom written using the Asyst programming environment.

Experimental protocol. Piglets were block randomized into four groups (n=8 each). In three groups, hypoxemia was induced by ventilating the piglets with an F_iO_2 of 0.10 by increasing the concentration of inhaled nitrogen gas. The F_iO_2 was adjusted as necessary from 0.07 to 0.15 as tolerated by the piglet, to obtain a P_aO_2 of 20-40 mmHg for 2 hours. Following

hypoxia, piglets were resuscitated with an F_iO_2 of 0.21 (21% group), 0.50 (50% group), or 1.0 (100% group) for 1 hour, followed by 0.21 for 3 hours. A control group of piglets was ventilated and oxygenated at an F_iO_2 of 0.21 for the 6 hours of study, with no period of hypoxia or reoxygenation. At the end of the experiment, piglets were euthanized with a bolus of 100 mg/kg pentobarbital. Hemodynamic variables were calculated as shown in Appendix 1 (page 177).

Blood and tissue collection. Arterial blood samples were drawn and immediately processed (Stat Profile, Nova Biomedical, Waltham, MA) at baseline, 120 minutes of hypoxia, and 30 and 240 minutes of reoxygenation. Immediately after the piglets were euthanized, the right lung was removed and snap-frozen in liquid nitrogen and stored at –80°C until biochemical analysis.

Measurement of lung glutathione level. Levels of total and oxidized glutathione (GSH) in lung tissue were measured using a GSH assay kit (catalogue no. 703002, Cayman Chemical, Ann Arbor, MI). Frozen samples of lung were homogenized (100 mg/mL) with buffer containing 0.2 M 2-N-morpholino ethanesulphonic acid, 50 mM phosphate, and 1 mM EDTA, pH 6.0. Homogenates were centrifuged at 10 000 g for 15 minutes at 4°C, and the supernatant was collected and deproteinated with 10% metaphosphoric acid and 4 M triethanolamine, to avoid interference from sulfhydryl groups on proteins in the sample. A colorimetric microplate assay was performed by adding GSH reductase, $NADP^+$, and 5,5'-dithiobis-2-nitrobenzoic acid to the sample. The absorbance was measured after 25 minutes at 405 nm with a

spectrophotometer (Bio-Rad 550 microplate reader, Hercules, CA), and the total GSH concentration was calculated from a standard curve. To measure oxidized glutathione (GSSG), deproteinated samples were incubated at room temperature for one hour with 1 M 2-vinylpyridine to completely derivatize the reduced GSH in the sample, and the colorimetric assay was carried out as above. Reduced GSH was calculated by subtracting GSSG from total GSH, and GSH redox status (GSSG:GSH) was obtained by calculating the oxidized-to-reduced GSH ratio.

Measurement of lung myeloperoxidase activity. Lung myeloperoxidase (MPO) activity was assayed using a modification of previously described procedures [19-21]. Frozen lung tissue was homogenized (200 mg/mL) in 0.5% hexadecyltrimethyl ammonium bromide with 10 mM potassium phosphate, pH 7.0. The homogenate was sonicated for 10 seconds, freeze-thawed 3 times in liquid nitrogen, sonicated again for 10 seconds, then centrifuged at 18 000 g for 30 minutes at 4°C. The assay was carried out by adding 200 uL of 1.6 mM tetramethylbenzidine containing 0.1 mM H_2O_2 to 10 uL of the homogenate, and reading the absorbance every 40 seconds for 2 minutes at 620 nm (Bio-Rad 550 microplate reader). Myeloperoxidase activity was calculated from the slope of the absorbance curve for each sample, and expressed as a percentage of control mean.

Measurement of lung lipid peroxidation. The concentration of lipid hydroperoxides (LOOH) in lung tissue was measured using a commercially available colorimetric assay (Lipid Peroxidation Assay Kit II, catalogue no. 437638, Calbiochem, San Diego, CA). Frozen lung tissue was homogenized

(100 mg/mL) in 4 mM butylated hydroxytoluene (BHT) in phosphate-buffered saline (with 1:100 acetonitrile to dissolve BHT). Catalase was added to the sample to eliminate interference by hydrogen peroxide, then ferrous ammonium sulfate and an indicator dye (xylene orange) were added, allowing LOOH to oxidize ferrous ions to ferric ions, which form a stable, coloured complex with xylene orange. Absorbance was read with the Bio-Rad 550 microplate reader at 550 nm. Background absorbance was measured for each sample by performing the assay with the addition of tris(2-carboxyethyl)phosphine which reduces LOOH to alcohols, and subtracting this from the absorbance without the reducing agent, to obtain the total LOOH concentration.

Statistical analysis. Results are expressed as mean ± standard error of the mean. The hemodynamic variables were analyzed by two-way repeated measures (RM) analysis of variance (ANOVA) followed by one-way RM ANOVA or one-way ANOVA to determine differences within groups over time or between groups at each timepoint, respectively, (SPSS 11.0.1, SPSS Inc., Chicago, IL, and Sigma Stat 2.0, Jandel Corp., San Rafael, CA). ANOVA was carried out as above on ranks if tests of normality or equal variances failed. Post-hoc testing using the Tukey method for one-way ANOVA and RM ANOVA or Dunnett's method for ANOVA on ranks was used for pairwise comparisons. One-way ANOVA with post-hoc LSD testing was used to determine differences in tissue biochemical results. Pearson correlation analysis was used to assess the relationship between hemodynamic or biochemical variables. Significance was defined as $p<0.05$.

RESULTS

Acid base status and hemodynamics during hypoxia and reoxygenation. Following 2 hours of hypoxia, animals developed a metabolic acidosis (pH 7.06 ± 0.04 vs. 7.44 ± 0.01 in controls, $p<0.001$), hypotension (MAP 27 ± 3 to 29 ± 1 mmHg vs. 68 ± 4 mmHg in controls, $p<0.001$) , and cardiogenic shock (cardiac index 54 ± 7 to 67 ± 5 $mL \cdot min^{-1} \cdot kg^{-1}$ vs. 145 ± 13 $mL \cdot min^{-1} \cdot kg^{-1}$ in controls, $p<0.001$). Hemodynamic variables recovered rapidly during reoxygenation, and by the end of reoxygenation, hemodynamics and acid-base status were similar to control values.

Oxygenation. Arterial oxygen tension (P_aO_2) decreased to less than half of control values in hypoxic piglets, and arterial oxygen content (C_aO_2) decreased to 10% of control values, as shown in figure 7-1. At 30 minutes of reoxygenation, P_aO_2 increased significantly in piglets reoxygenated with 50% or 100% oxygen compared to 21% and controls, however no differences were found at 240 minutes of reoxygenation. Measures of C_aO_2 immediately recovered and were not significantly different from control values in all experimental groups upon reoxygenation, although C_aO_2 was greater in the 50% and 100% groups compared to 21% at 30 minutes of reoxygenation, but not at 240 minutes of reoxygenation.

Lung glutathione levels. Total, reduced and oxidized GSH levels are shown in table 7-1. There were no significant differences in GSH levels among the experimental groups. Figure 7-2 shows the GSH redox status in lung, in which the 50% group but not the 100% group had a significantly higher GSSG:GSH ratio than the 21% group.

TABLE 7-1. GLUTATHIONE LEVELS IN PIGLET LUNG FOLLOWING HYPOXIA AND REOXYGENATION.

Group	Total glutathione µmol/g protein	Reduced glutathione µmol/g protein	Oxidized glutathione (GSSG) µmol/g protein
Control	30.0 ± 1.8	26.9 ± 1.6	3.1 ± 0.8
21 %	29.0 ± 1.9	26.2 ± 1.7	2.8 ± 0.6
50 %	30.1 ± 2.0	27.5 ± 1.8	3.2 ± 0.6
100 %	29.9 ± 1.6	26.9 ± 1.4	3.1 ± 0.7

Results are shown as mean ± SEM.

Lung myeloperoxidase activity. There were no significant differences in lung MPO activity in any of the study groups, as shown in figure 7-3.

Lipid peroxidation in lung. Lipid hydroperoxide levels in piglets exposed to hypoxia-reoxygenation were slightly but not significantly greater than in control piglets (1.06 ± 0.24 µmol LOOH/g protein in controls). There were no differences between lung LOOH levels among the study groups (see figure 7-4).

Correlations. Arterial oxygen content or P_aO_2 during reoxygenation showed no correlation with lung GSH levels, MPO activity, or LOOH levels. There was a significant negative correlation between MPO activity and GSSG level ($r = -0.545$, $p<0.01$).

FIGURE 7-1(A). ARTERIAL OXYGEN TENSION

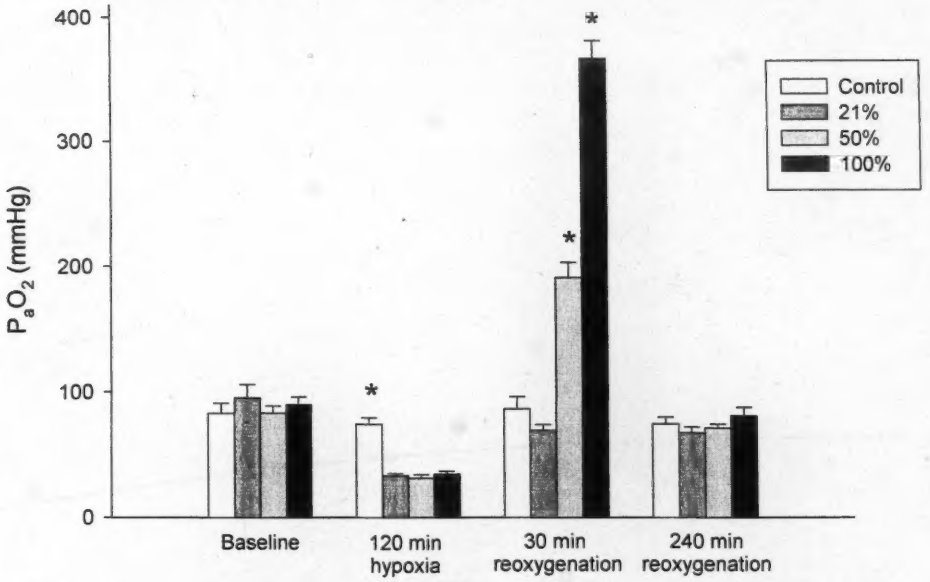
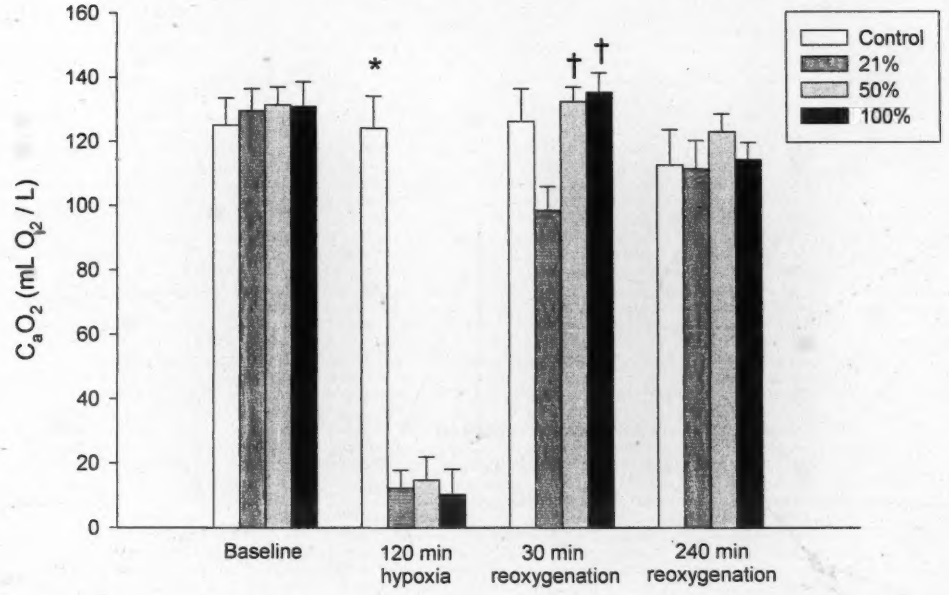
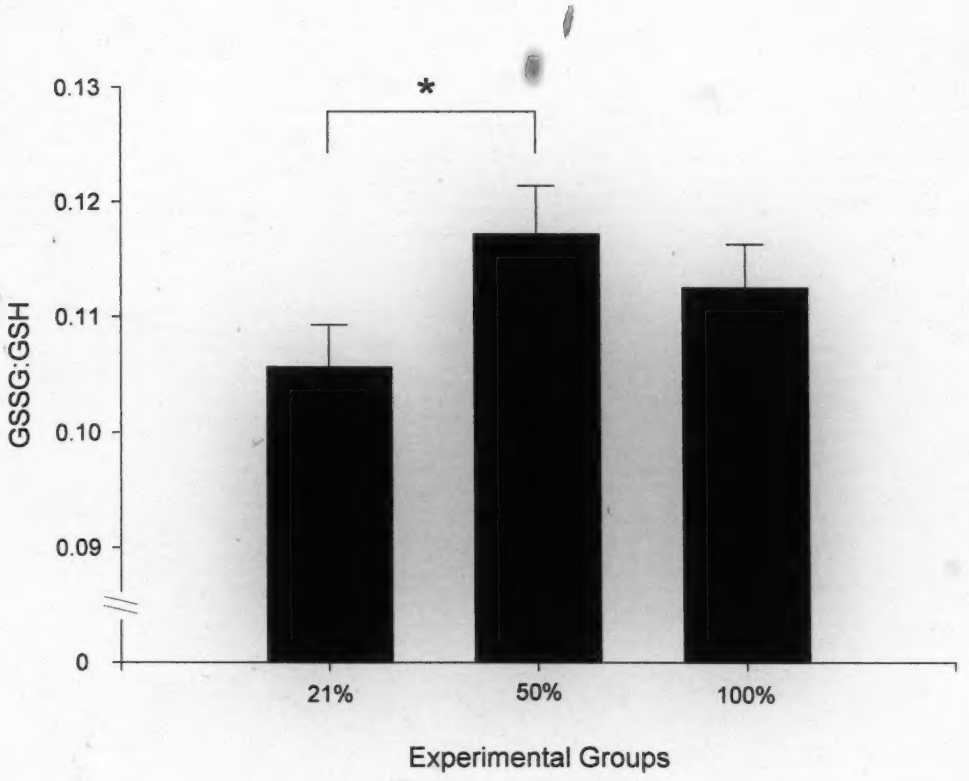


FIGURE 7-1(B). ARTERIAL OXYGEN CONTENT



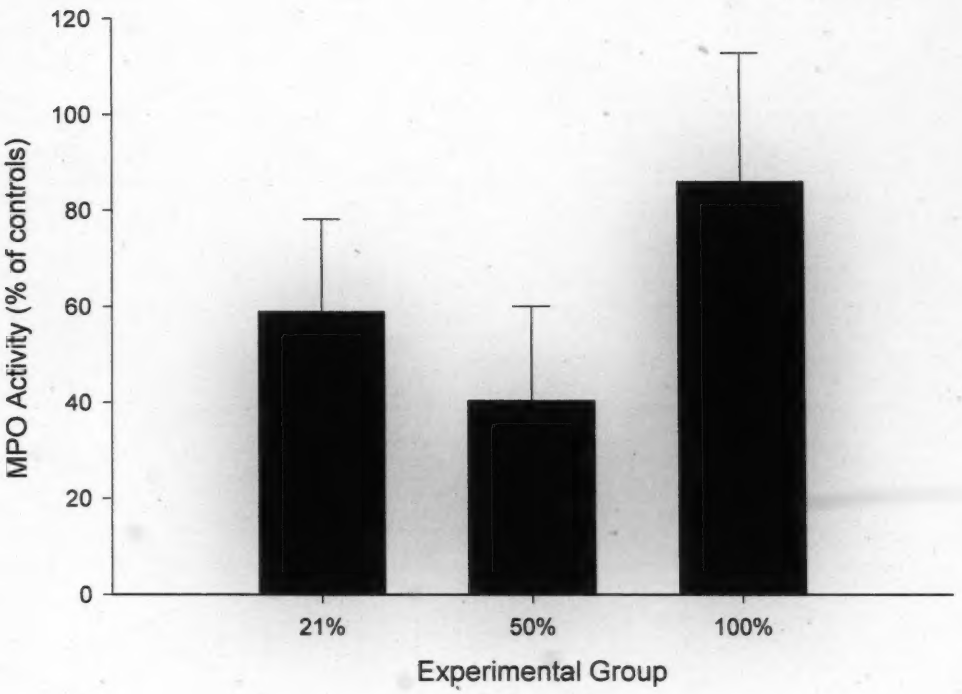
(A) Arterial oxygen tension (P_aO_2) and (B) content (C_aO_2) in newborn piglets during hypoxia and reoxygenation. Control piglets underwent no hypoxia-reoxygenation. * $P<0.001$ vs. all other groups. † $P<0.05$ vs. 21% group.

FIGURE 7-2. GLUTATHIONE REDOX RATIO IN LUNG



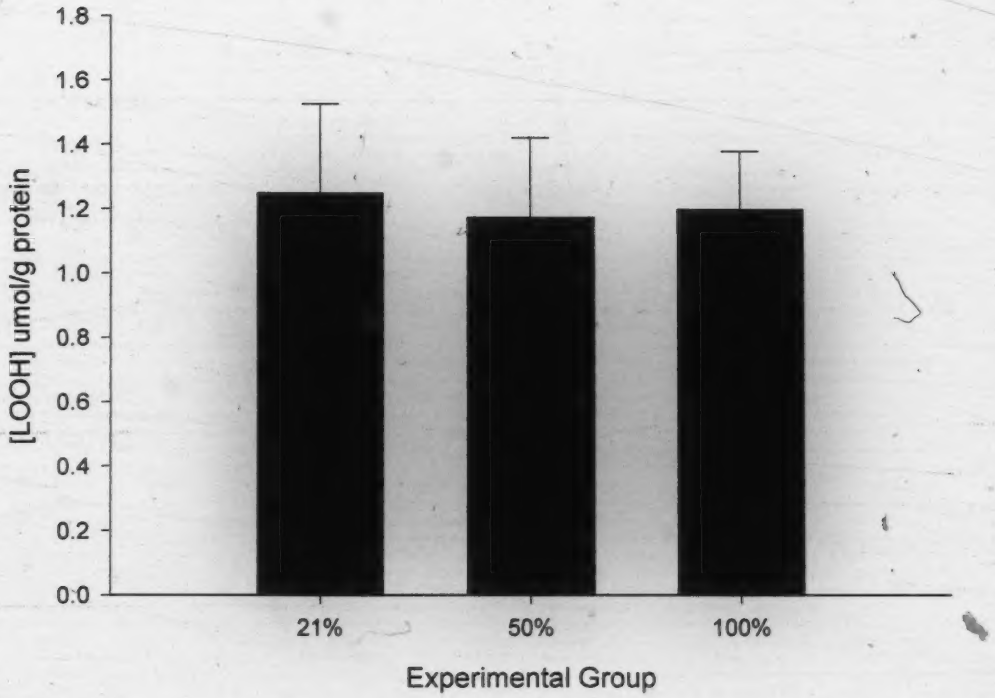
Glutathione redox ratio (GSSG:GSH) in newborn piglet lung following hypoxia and reoxygenation with different oxygen concentrations. (*P<0.05)

FIGURE 7-3. MYELOPEROXIDASE ACTIVITY IN LUNG



Myeloperoxidase (MPO) activity in newborn piglet lung following hypoxia and reoxygenation with different oxygen concentrations.

FIGURE 7-4. LIPID HYDROPEROXIDES IN LUNG



Lipid hydroperoxide (LOOH) content in newborn piglet lung following hypoxia and reoxygenation with different oxygen concentrations.

DISCUSSION

Prolonged exposure to high oxygen concentrations leads to acute pulmonary injury and OFR activation, and predisposes to CLD.[1] Few previous studies have addressed the effects of a brief period of hyperoxia, during resuscitation following severe asphyxia, on lung OFR activity. Our study has examined three indices of free radical activity in the lung. We have shown that the GSH redox status, or GSSG:GSH ratio, is greater in piglets resuscitated with 50% oxygen compared to 21% oxygen, indicating an increase in oxidative stress in the 50% group. In piglets resuscitated with 100% oxygen however, GSSG:GSH was not significantly higher than in the 21% group. Myeloperoxidase activity, a marker of neutrophil infiltration and activation, was not significantly different in any group. Lipid hydroperoxide concentration, a marker of lipid peroxidation, was slightly but not significantly elevated in piglets following hypoxia-reoxygenation compared to controls, but there were no differences between any of the resuscitated groups.

Glutathione is one of the most important endogenous antioxidants, and the GSH redox ratio has been used as a marker of oxidative stress.[18,22,23] Oxygen free radicals react directly with GSH, and form GSSG catalyzed by GSH peroxidase, thereby detoxifying the OFRs and protecting the cell from further oxidative damage.[24] Study of rats, rabbits, guinea pigs and hamsters has shown that GSH peroxidase levels in lungs of premature animals are lower than full term animals,[25] but lung GSH peroxidase levels in humans do not change with gestation and up to 3 months post partum.[26,27] Although we are

aware of no direct comparisons of newborn to adult lung GSH levels, it has been suggested that newborns, and especially premature newborns, have immature antioxidant defences in their lungs, and thus may be more susceptible to oxygen toxicity.[7] Indeed, Frank and Sosenko[28] showed that premature newborn rabbits exposed to 90% oxygen for 48 hours showed no increase in antioxidant enzyme activity, and developed severe lung injury. In the present study, there was no significant increase in GSSG or GSH redox status in reoxygenated piglets compared to controls. This may be due in part to an inability to increase GSH peroxidase activity due to an immaturity of antioxidant defences in newborn piglets. In addition, our period of hyperoxia is relatively brief, 1 hour, followed by 3 hours of ventilation with room air as in controls, allowing cellular GSH stores to replenish.

Neutrophils have been demonstrated as mediators of hypoxia-reoxygenation injury in many organs, including lung, intestine, heart, kidney, brain, liver and muscle.[21,29-31] Oxygen free radicals indirectly mediate neutrophil infiltration and activation following hypoxia-reoxygenation, and further induce OFR generation by MPO and NADPH oxidase of the activated neutrophil.[30,32-34] Myeloperoxidase, a neutrophil-specific enzyme, converts hydrogen peroxide to the highly cytotoxic hypochlorous acid.[30,32] Johnson *et al.*[3] demonstrated that instillation of a hydrogen peroxide precursor plus MPO into rat lung caused severe acute lung injury. It is well-established that neutrophils accumulate in the lungs of infants with respiratory distress, and that neutrophil infiltration is an important contributor to the development of CLD,[35,36] although the role of MPO-derived oxidants *per se* has not been

established. Buss *et al.*[14] demonstrated that MPO activity in tracheal aspirates of premature infants did not correlate with the duration of oxygen exposure or F_iO_2 at the time of sampling, however they did show that MPO activity correlated with lipid peroxidation products and protein oxidation products. Our study has shown that MPO activity was not influenced by hypoxia-reoxygenation or the concentration of oxygen used. Interestingly, we have demonstrated a negative correlation between the level of GSSG and MPO activity. We speculate that in the acute setting, hydrogen peroxide generated by the hypoxanthine-xanthine oxidase system follows either the GSH peroxidase pathway and forms GSSG, or follows the MPO pathway. Thus initially, with increasing MPO activity, there may be less hydrogen peroxide to react with GSH to form GSSG. However with more prolonged oxidative stress, we expect that increasing MPO activity and formation of powerful oxidants will eventually lead to an increase in GSSG formation.

Plasma membranes, nuclear membranes and other organelles are the prime targets of OFR-induced lipid peroxidation, which can lead to changes in cellular permeability and ultimately cell lysis and death.[37] Ogihara *et al.*[13] demonstrated that an increase in plasma lipid peroxidation products at 24 hours of age in newborns was an independent predictor of the subsequent development of CLD. In contrast, Winterbourn *et al.*[38] found no difference in plasma lipid peroxidation markers at 7 and 28 days of age in premature infants with or without CLD, and Buss *et al.*[14] found no correlation between lipid peroxidation products in tracheal aspirates of premature newborns and the duration of supplemental oxygen exposure, or the F_iO_2 at the time the

sample was taken. Similarly in the current study, the concentration of oxygen used in resuscitation had no effect on lipid peroxidation in the lung.

In summary, our study has demonstrated that there is little difference in oxidative stress, MPO activity, and lipid peroxidation in the lungs of asphyxiated newborn piglets resuscitated with 21%, 50% or 100% oxygen. Furthermore, neither C_aO_2 nor P_aO_2 during resuscitation correlate with any of our measured free radical indices. In conclusion, we suggest that the oxygen concentration used during a brief period of neonatal resuscitation will likely not result in acute lung injury.

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CHAPTER 8

Improved Carotid Flow in Hypoxemic Newborn Piglets Reoxygenated with 21% Oxygen

INTRODUCTION

Perinatal asphyxia is the single most important cause of brain injury in newborns, which manifests as hypoxic-ischemic encephalopathy.[1] Hypoxic-ischemic encephalopathy occurs in approximately 72% of asphyxiated newborns,[2] and many neonates with moderate to severe encephalopathy go on to develop permanent neurodevelopmental sequelae.[3,4] Severe hypoxic-ischemic encephalopathy frequently has associated organ involvement, including heart (62%), lung (33%), kidney (28%), liver (22%), and intestine (6%).[5]

During perinatal asphyxia, cerebral blood flow and oxygenation may be compromised, leading to hypoxia-related cytotoxic edema in neurons. With prolonged ischemia, irreversible cell necrosis can result. With reoxygenation, the cellular energy deficit will temporarily be corrected, by resuming oxygen delivery to replenish ATP stores, however reoxygenation may trigger a chain of events leading to delayed neuronal death or damage due to brain swelling, as seen clinically in hypoxic-ischemic encephalopathy, or mitochondrial dysfunction.[1,6,7] Delayed neuronal death as a result of mitochondrial dysfunction has been attributed to the action of oxygen free radicals[7] originating in brain capillaries[8,9] which can impair the mitochondrial electron transport chain.[10]

Previous animal studies have examined the cerebral blood flow changes and neurologic effects of resuscitation with 21% versus 100% oxygen. Rootwelt *et al.*[11] demonstrated that regional cerebral blood flow to the forebrain, midbrain, brainstem, cerebellum, and cervical spinal cord

increased equally in hypoxemic newborn piglets resuscitated with 21% or 100% oxygen, and further reported no difference in histologic evidence of brain damage in the cerebral cortex, cerebellum and hippocampus between the two groups.[12] Temesvari *et al.*[13] demonstrated that asphyxiated newborn piglets resuscitated with 100% oxygen had significantly worse early clinical neurologic outcome compared to both 21% piglets and controls. However, cerebral histopathology revealed marked damage of similar severity in both asphyxiated groups.

Evidence of cerebral OFR activity has been examined in newborn animals resuscitated with 21% versus 100% oxygen. Poulsen *et al.*[14] examined hypoxanthine concentrations, a marker of hypoxia and ATP breakdown, in the cerebral cortex of newborn piglets. They found a higher level of hypoxanthine in hypoxemic piglets resuscitated with 100% compared to 21%, and speculated that there may be a more severe impairment of energy metabolism in the cerebral cortex, or increased blood brain barrier damage and leak of hypoxanthine into the brain, in the 100% group compared to 21%. Kutzsche *et al.*[15] found higher hydrogen peroxide concentrations in neutrophils in the cerebral circulation in 100%-resuscitated newborn piglets compared to 21%.

These studies suggest that different concentrations of oxygen used in resuscitation may have important cerebral effects. In our study, the objective was to determine the changes in carotid blood flow, vascular resistance, and oxygen delivery during hypoxia and reoxygenation with 21%, 50% or 100% oxygen in newborn piglets.

METHODS

The methods have been previously described in chapters 4, 5 and 6. In addition, a 2-mm Transonic™ flow probe (2S171, Transonic Systems Inc., Ithica, NY) was placed around the left common carotid artery for continuous monitoring of left carotid blood flow during the experimental protocol. Carotid flow index, carotid vascular resistance index and carotid and systemic oxygen delivery index were calculated as shown in Appendix 1 (page 177).

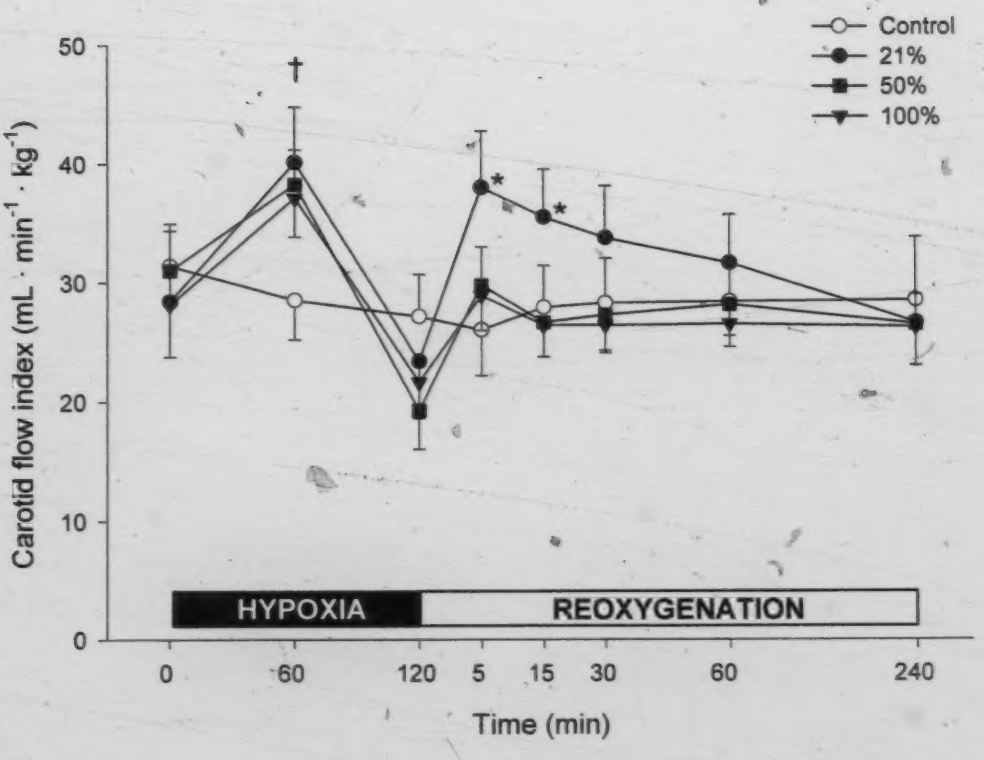
RESULTS

Carotid flow index. During early hypoxia carotid flow increased significantly compared to baseline values, and compared to normoxic controls, as shown in figure 8-1. After two hours of hypoxia, carotid flow decreased back to baseline values. During the first 15 minutes of reoxygenation, carotid flow increased significantly in piglets resuscitated with 21% oxygen.

Carotid vascular resistance index. The increases in carotid flow were accompanied by decreases in vascular resistance, as shown in figure 8-2. Carotid vascular resistance index decreased throughout hypoxia, and in the 21% group, carotid resistance was significantly below baseline values during the entire reoxygenation period.

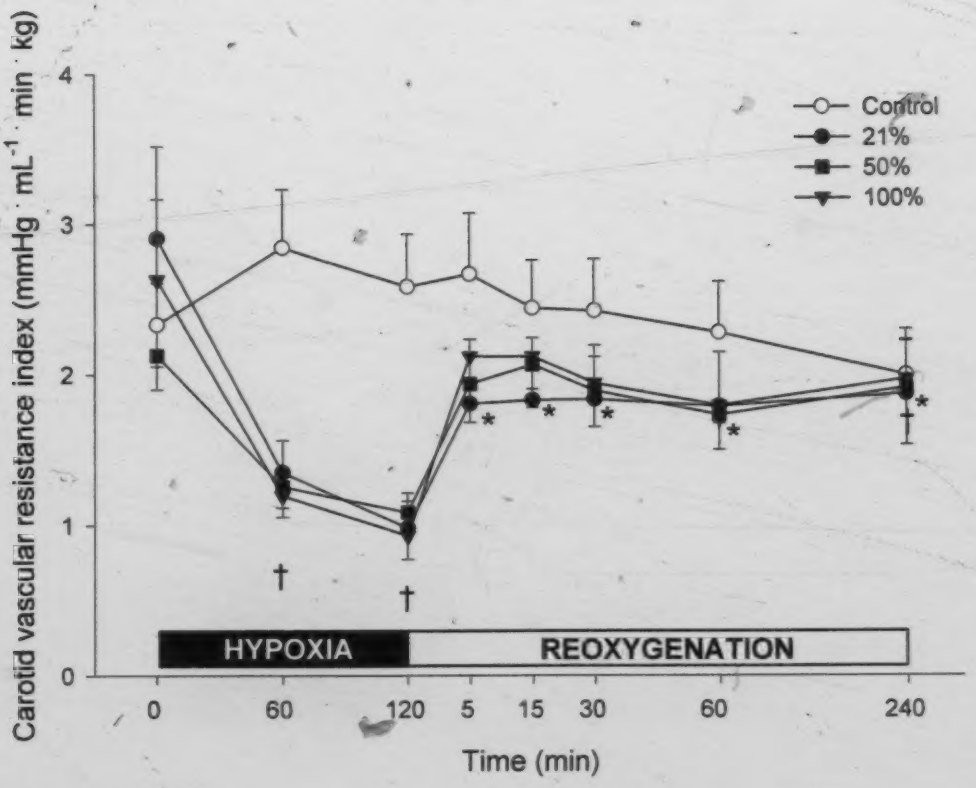
Carotid and systemic oxygen delivery. During hypoxia, carotid oxygen delivery index decreased to 9% ± 3%, and total oxygen delivery index decreased to 4% ± 1% of baseline values. Upon reoxygenation, carotid and systemic oxygen delivery immediately recovered to baseline and control values in all experimental groups, except in the 100% group systemic oxygen delivery was transiently greater than controls at 5 minutes of reoxygenation, (see figures 8-3 and 8-4).

FIGURE 8-1. CAROTID ARTERY FLOW



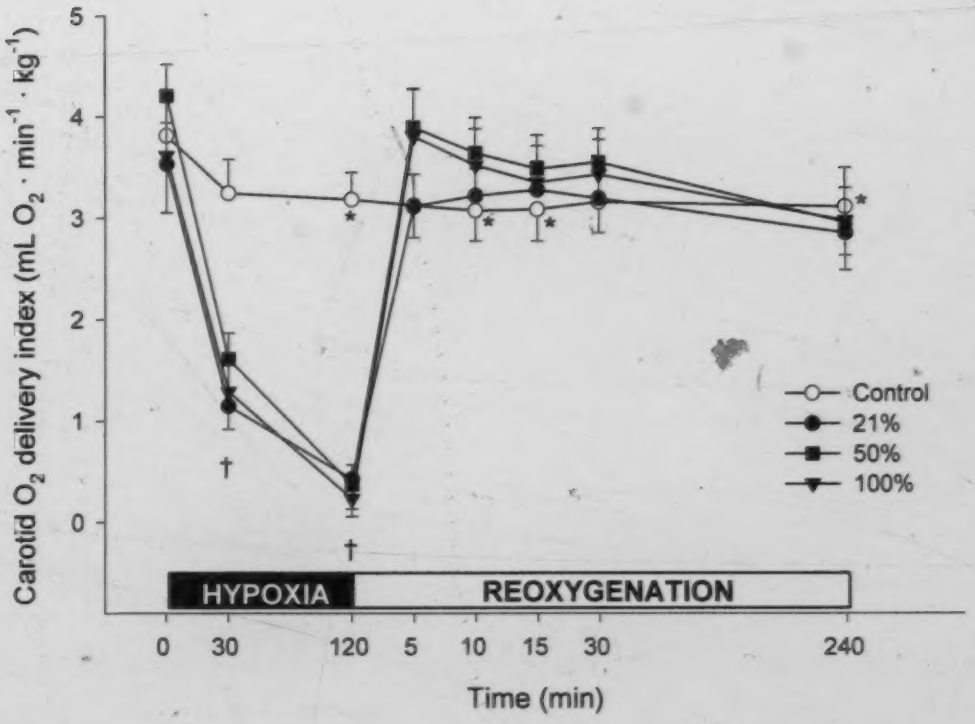
Carotid artery flow index during hypoxia and reoxygenation with different oxygen concentrations. Control piglets (sham operated) underwent no hypoxia or reoxygenation. † $P<0.05$ hypoxic groups vs. controls and baseline. *$P<0.05$ vs. baseline.

FIGURE 8-2. CAROTID VASCULAR RESISTANCE



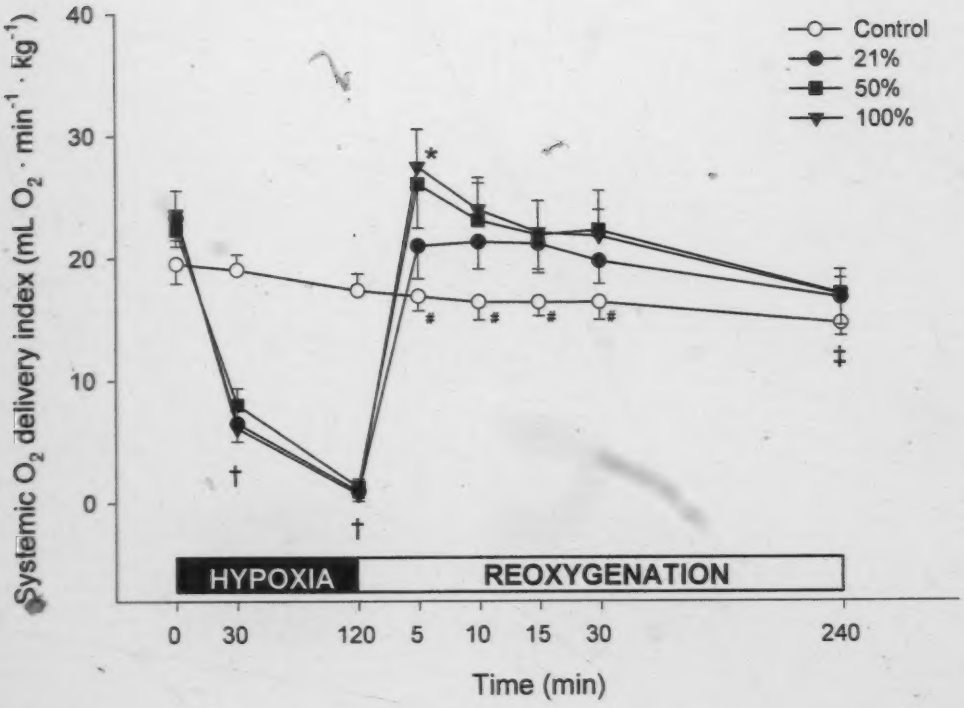
Carotid vascular resistance index during hypoxia and reoxygenation with different oxygen concentrations. Control piglets (sham operated) underwent no hypoxia or reoxygenation. † P<0.05 all hypoxic groups vs. controls and baseline. * P<0.05 21% group vs. baseline.

FIGURE 8-3. CAROTID OXYGEN DELIVERY



Carotid oxygen delivery index during hypoxia and reoxygenation with different oxygen concentrations. Control piglets (sham operated) underwent no hypoxia or reoxygenation. † P<0.001 all hypoxic groups vs. controls and baseline. * P<0.05 control vs. baseline.

FIGURE 8-4. SYSTEMIC OXYGEN DELIVERY



Systemic oxygen delivery index during hypoxia and reoxygenation with different oxygen concentrations. Control piglets (sham operated) underwent no hypoxia or reoxygenation. † P<0.001 all hypoxic groups vs. controls and baseline. * P<0.05 100% group vs. control. # P<0.05 control vs. baseline. ‡ P<0.05 all groups vs. baseline.

DISCUSSION

In piglets subjected to hypoxemia, we observed a cerebral protective effect, with carotid flow never decreasing significantly below baseline or control values. This effect was mediated by a significant decrease in carotid vascular resistance throughout the hypoxic phase. Cerebral vascular changes during asphyxia have been previously well-documented. During hypoxemia and acidosis, circulatory adjustments occur which differentially effect blood flow and oxygenation to different organs, preferentially shunting blood to the more vital organs for survival.[16-18] Local vascular effects cause a hypoxemia-induced vasodilation in the cerebral vasculature, which facilitates redistribution of blood flow to the brain which has the greatest metabolic requirements.[16] Alpha-adrenergic stimulation and chemoreceptor stimulation further aide flow redistribution by causing local vasoconstriction in the splanchnic, renal and skeletal muscle vasculature.[16,19] This cerebral protective effect however has a limited capacity, and with severe hypoxemia, the redistribution of blood flow may be inadequate to meet the brain's metabolic needs. In the current study, in spite of the increased flow observed initially, carotid oxygen delivery was severely compromised throughout hypoxia. Furthermore, carotid blood flow elevation was not sustained, in spite of a sustained carotid vasodilation during hypoxia, due to a substantial decrease in cardiac output after 2 hours of hypoxia.

Immediately upon reoxygenation, we observed a transient increase in carotid flow in piglets resuscitated with 21% oxygen. Interestingly, the carotid

vascular resistance recovered partially yet remained significantly reduced in piglets upon reoxygenation with 21% oxygen, in contrast to the recovery of the carotid vascular resistance to baseline values in piglets resuscitated with 50% or 100% oxygen. Nonetheless, carotid oxygen delivery immediately recovered in all experimental groups. Rootwelt *et al.*[11] previously reported an increase in cerebral blood flow during reoxygenation in hypoxemic piglets resuscitated with either 21% or 100% oxygen. In their study, the degree of hypoxemia was comparable to the current study, although the duration of hypoxemia was shorter (60 minutes). We suggest that the difference in severity of hypoxic injury to the cerebral vasculature between the studies may explain the different observations upon reoxygenation. Furthermore, many studies have demonstrated that oxygen free radicals are important mediators of vasoconstriction in the cerebral,[20] coronary,[21] pulmonary,[22-24] splanchnic,[25] and renal [26] vasculatures and in the aorta.[27] We speculate that, as demonstrated in our experimental model, resuscitation of severely hypoxemic piglets with higher concentrations of oxygen resulted in a relative increase in vascular tone compared to the 21% group, as a result of an increase in oxygen free radical generation.

In conclusion, this study has demonstrated that resuscitation of asphyxiated newborn piglets with 21% oxygen results in improved carotid blood flow with a decrease in carotid vascular resistance.

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CHAPTER 9

Renal Hemodynamics During Resuscitation with 21%, 50% or 100% Oxygen

INTRODUCTION

Acute renal failure develops in 9 to 30% of severely asphyxiated neonates.[1,2] Luciano *et al.*[2] demonstrated a decrease in renal blood flow velocity following perinatal asphyxia compared to healthy control neonates, and further showed that asphyxiated neonates with acute renal failure had significantly decreased blood flow compared to both healthy neonates and asphyxiated neonates without renal failure. In newborn animal models, renal blood flow has been shown to decrease significantly with asphyxia in piglets [3] and lambs,[4] but not in rabbits.[5] During reoxygenation, renal blood flow has been shown to recover gradually in newborn piglets, whether 21% or 100% oxygen is used.[6]

The objective of our study was to determine the changes in renal blood flow, vascular resistance, and oxygen delivery during hypoxia and reoxygenation with 21%, 50% or 100% oxygen.

METHODS

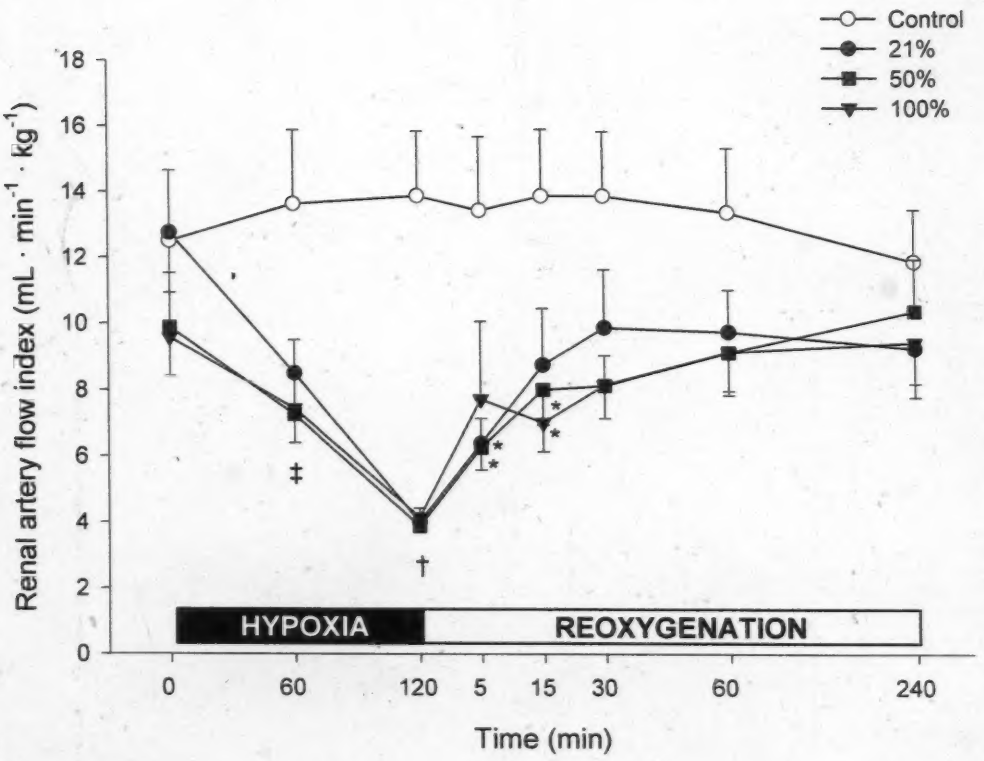
The methods have been previously described in chapters 4, 5 and 6. Briefly, the left renal artery was isolated in the retroperitoneum via a left flank incision, and a 2-mm Transonic™ flow probe (2SB694, Transonic Systems Inc., Ithica, NY) was placed around the renal artery for continuous monitoring of left renal blood flow during the experimental protocol. Renal artery flow index, renal vascular resistance index, and renal oxygen delivery index were calculated as shown in Appendix 1 (page 177).

RESULTS

Effects of hypoxia. During hypoxia, renal blood flow index decreased to 29% ± 1% and renal oxygen delivery index decreased to 3% ± 1% of control values, as shown in figures 9-1 and 9-2, respectively. Renal vascular resistance did not change significantly during hypoxia, as shown in figure 9-3.

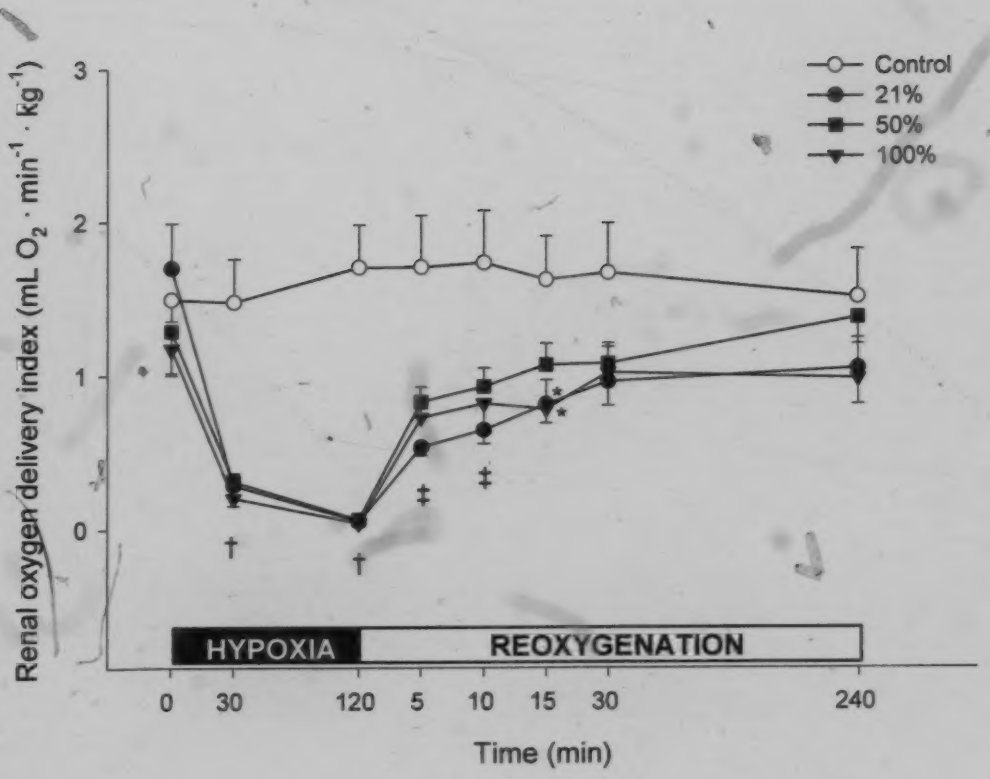
Effects of reoxygenation. Renal blood flow returned to values not significantly different from controls by 15 minutes of reoxygenation in the 21% group, and by 30 minutes of reoxygenation in the 50% and 100% groups (figure 9-1). Renal oxygen delivery remained decreased in the first 10 minutes of reoxygenation in the 50% group, and during the first 15 minutes of reoxygenation in the 21% and 100% groups (figure 9-2). Renal vascular resistance increased significantly at 5 minutes of reoxygenation (all reoxygenated piglets versus controls, $p<0.05$, figure 9-3), although there were no differences between the experimental groups.

FIGURE 9-1. RENAL ARTERY FLOW



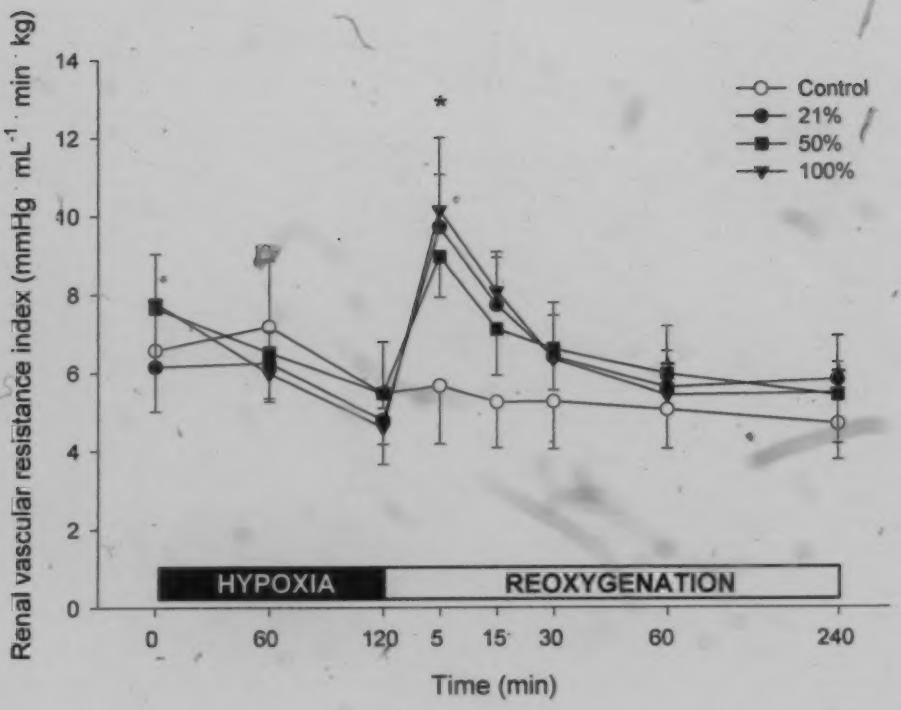
Renal artery flow index during hypoxia and reoxygenation with different oxygen concentrations. Control piglets (sham operated) underwent no hypoxia or reoxygenation. ‡ P<0.005 and † P<0.001 all hypoxic groups vs. controls. *P<0.05 vs. control.

FIGURE 9-2. RENAL ARTERY OXYGEN DELIVERY



Renal artery oxygen delivery index during hypoxia and reoxygenation with different oxygen concentrations. Control piglets (sham operated) underwent no hypoxia or reoxygenation. † $P<0.001$ and ‡ $P<0.05$ all hypoxic groups vs. controls. *$P<0.05$ vs. control.

FIGURE 9-3. RENAL VASCULAR RESISTANCE



Renal vascular resistance index during hypoxia and reoxygenation with different oxygen concentrations. Control piglets (sham operated) underwent no hypoxia or reoxygenation. *P<0.05 all hypoxic groups vs. control.

DISCUSSION

Our study has shown that renal blood flow and oxygenation are severely depressed by asphyxia in newborn piglets, and both are slow to recover upon reoxygenation, with renal blood flow and oxygen delivery recovering slightly earlier in the 21% and 50% groups, respectively. Although there was no change in renal vascular resistance with hypoxia, upon reoxygenation, there was a transient, significant increase in vascular resistance. These results are in contrast to those observed in our studies in the carotid (chapter 8) and mesenteric (chapter 6) vasculature, whereby vascular resistance decreased during reoxygenation, and blood flow immediately recovered or increased above baseline values. Similar findings were demonstrated by Rootwelt *et al.*[6] in hypoxemic newborn piglets resuscitated with 21% or 100% oxygen, wherein renal blood flow remained below baseline values during the first 20 minutes of reoxygenation in both groups, while blood flow in the intestine, muscle and heart recovered to above baseline values immediately. Similarly, Luciano *et al.*[2] demonstrated a decrease in renal blood flow in neonates following perinatal asphyxia as measured by doppler blood flow velocity, and a greater decrease in blood flow was observed in infants developing acute renal failure after perinatal asphyxia. Creatinine clearance, fractional sodium excretion, plasma creatinine and renal blood flow normalized within the first two weeks of life in all infants.

It has been well-established that blood flow redistribution occurs during

hypoxemia, whereby blood flow is preferentially shunted to the more metabolically active and vital organs, namely the brain and the heart, at the expense of the kidneys, intestines, spleen, muscle and skin. Local vasodilation has been shown to occur in response to hypoxemia predominantly in the cerebral and coronary circulations, while the renal vasculature does not vasodilate even at very low oxygen tension.[7-9] The current study has demonstrated an ongoing blood flow redistribution during reoxygenation, which may occur by similar mechanisms as during hypoxemia, namely selective local vasodilation, alpha-adrenergic stimulation, and chemoreceptor stimulation, all of which favor an increase in cerebral and coronary blood flow and a decrease in renal blood flow.[8] Furthermore, we speculate that the renal vasculature may be more sensitive to the vasoconstricting effects of oxygen free radicals. Oxygen free radicals have been shown to mediate vasoconstriction in the renal vasculature,[10] as well as cerebral,[11] coronary,[12] pulmonary,[13-15] and splanchnic[16] circulations and in the aorta,[17] although we are not aware of any comparative study indicating a greater effect of oxygen free radicals in the renal circulation.

In conclusion, in this study we have demonstrated profound effects of hypoxemia on renal blood flow and oxygen delivery. With reoxygenation, we have shown an increase in renal vascular resistance in piglets resuscitated with 21%, 50% and 100% oxygen, which resulted in a slow recovery of renal blood flow during the reoxygenation period.

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CHAPTER 10

Conclusions

In the newborn, perinatal asphyxia and subsequent resuscitation invariably creates a hypoxia-reoxygenation process, with tissue injury mediated in part by oxygen free radicals. This research has endeavored to determine whether the concentration of oxygen used in resuscitation following severe asphyxia has an impact on functional, biochemical and histologic outcome.

Our study has demonstrated significant hemodynamic changes during early reoxygenation, with improved cardiac index and regional flow in the carotid, mesenteric and renal arteries in piglets resuscitated with 21% oxygen. This may be due to improved cardiac function. We also speculate that higher oxygen concentrations used in resuscitation caused a relative increase in vascular tone, mediated in part by oxygen free radicals. Indeed, a decrease in vascular resistance was observed systemically and in the carotid and mesenteric arteries with the use of 21% oxygen during resuscitation. Importantly, mean arterial blood pressure recovered immediately in spite of this decreased vascular resistance. Thus we conclude that 21% oxygen is indeed effective at systemic and regional hemodynamic recovery following severe asphyxia, and may even be superior to higher concentrations of oxygen. A potential disadvantage of 21% oxygen resuscitation, however, is the prolonged elevation in pulmonary arterial pressure, which may predispose to persistence of the fetal circulation and worsened hypoxia. Therefore in susceptible newborns and those with congenital pulmonary or cardiac anomalies, the use of lower oxygen concentrations may not be appropriate. Furthermore, the changes we

observed in both systemic and pulmonary hemodynamics were of short duration, and in our acute experimental model, we did not study the effects, if any, that these transient changes in blood flow may have had in the long term.

Glutathione is one of the most important cellular antioxidants, and our study has demonstrated that increasing oxygen exposure caused significant changes in tissue glutathione status. In the heart, glutathione stores were depleted with 100% oxygen resuscitation. This may have detrimental hemodynamic effects, as we further demonstrated that cardiac function positively correlated with, and may be dependent upon, the myocardial total glutathione level. In the small intestine evidence of oxidative stress was observed with increasing superior mesenteric oxygen delivery, and further, histologic lesions similar to necrotizing enterocolitis were found in piglets resuscitated with 100% oxygen, indicating resuscitation with high oxygen concentrations may increase the risk of intestinal reoxygenation injury, mediated by oxygen free radicals. Thus in the heart and the small intestine, 100% oxygen resuscitation may be detrimental, while 21% oxygen had no apparent adverse effects. In the lung however, we did not observe oxygen-dependent changes in markers of oxidative stress, neutrophil activation, or oxygen free radical activity.

In summary, this research has helped to increase our understanding of the physiologic changes that occur during graded reoxygenation in the acute setting, and future studies in a chronic animal model will help to determine if these acute changes have a significant impact in the longer

term. Furthermore, we have demonstrated the importance of the glutathione status as an indicator of oxidative stress in newborn resuscitation, and future studies may be aimed at examining the potential therapeutic role of glutathione or other antioxidants and free radical scavengers during perinatal asphyxia and reoxygenation. Finally, this research strengthens the emerging literature indicating that the use of 21% oxygen during newborn resuscitation is both safe and effective, and a very reasonable alternative to 100% oxygen.

APPENDIX 1
Hemodynamic Calculations

TABLE A-1. HEMODYNAMIC CALCULATIONS

Hemodynamic variable	Calculation
Cardiac index (CI)	Cardiac output ÷ weight
SMA flow index	SMA flow ÷ weight
Carotid artery flow index	Carotid flow ÷ weight
Renal artery flow index	Renal flow ÷ weight
Stroke volume index	CI ÷ HR
Systemic vascular resistance index	(MAP – CVP) ÷ CI
Pulmonary vascular resistance index	PAP ÷ CI
SMA vascular resistance index	MAP ÷ SMA flow index
Carotid vascular resistance index	(MAP – CVP) ÷ Carotid flow index
Renal vascular resistance index	(MAP – CVP) ÷ Renal flow index
Arterial oxygen content (C_aO_2)	(1.39 x Hgb x S_aO_2) + (0.03 x P_aO_2)
Systemic delivery index	C_aO_2 x CI
SMA oxygen delivery index	C_aO_2 x SMA flow index
Carotid oxygen delivery index	C_aO_2 x Carotid flow index
Renal oxygen delivery index	C_aO_2 x Renal flow index

HR=heart rate, MAP=mean arterial pressure, PAP=mean pulmonary artery pressure, SMA=superior mesenteric artery, Hgb=hemoglobin, S_aO_2=arterial oxygen saturation

APPENDIX 2
Surgical Procedure Photographs

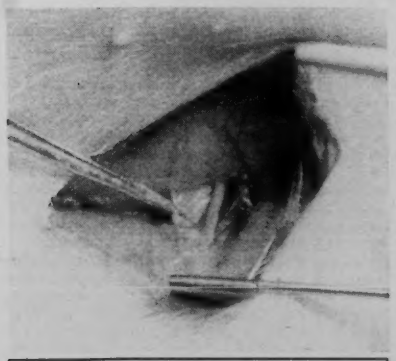

Figure A-1. Right femoral artery and vein dissection.

Figure A-2. Femoral arterial and venous catheters.

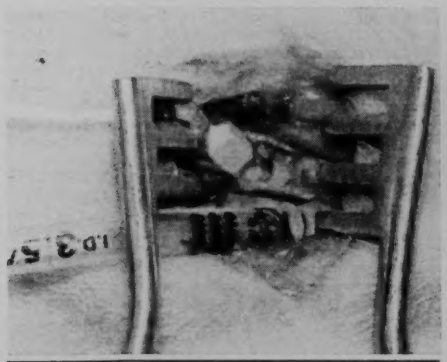

Figure A-3. Tracheostomy and left carotid flow probe.

Figure A-4. SMA dissection in retroperitoneum.

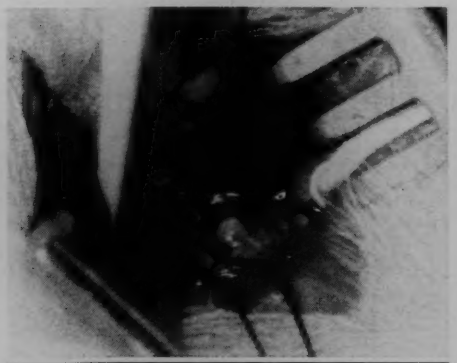

Figure A-5. Left renal artery (looped), and SMA flow probe.

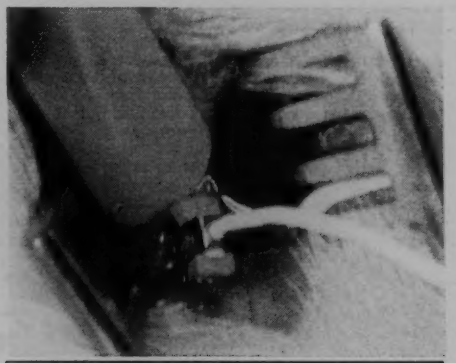

Figure A-6. Left renal artery flow probe.

Figure A-7. Main pulmonary artery with purse-string suture.

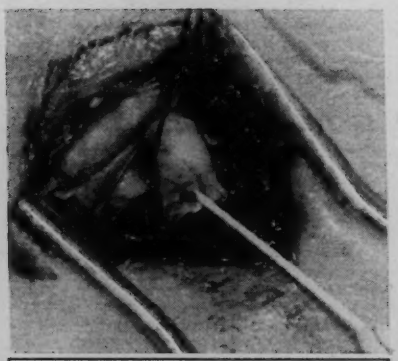

Figure A-8. Pulmonary artery pressure catheter.

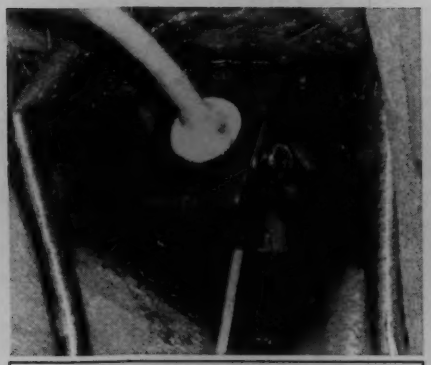

Figure A-9. Pulmonary artery flow probe.

Figure A-10. Laboratory setup and monitors.